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THE IMMUNITY REACTIONS AGAINST CULTIVATED *SPIRONEMA PALLIDUM* OF GENERAL PARALYTICS TREATED BY INDUCED MALARIA.

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THE years that have elapsed since general paralysis was first treated by artificially induced malaria, and the numerous reports which have been published during that time, all seem to confirm the original belief that this treatment produced a temporary (or possibly a permanent) remission or amelioration of the clinical symptoms. This improvement, from the work of Brutsch and Bahr (1929), Wilson (1928), Geary (1929) and others, seems primarily due to the destruction of the *Spironema pallidum*, and secondarily to repair of the infected tissue, but the mechanism of this destruction is unknown.

Whether the human body develops immune bodies to even cultured *Spironema pallidum* is still extremely doubtful. There seems to be some unanimity in the opinion that the serum of an actively immunized animal possesses agglutinating and spirochæticidal properties against the specific strain of *Spironema pallidum*, but when human serum is investigated the results are very conflicting. Kolmer (1913) could find no demonstrable amounts of agglutinin in any type of human serum, whilst Kissmeyer (1915), and Kolmer, Broadwell and Matsumani (1916), obtained positive agglutinations in relatively high titres in the sera of syphilitics. The evidence, both for and against human serum possessing spirochæticidal properties, is as equally conflicting; the results seemingly being dependent on the strain of spirochæte used.

This discordance of opinion necessitates first the investigation of non-treated sera, either with a positive Wassermann reaction or a negative reaction, before any comparision can be made with the results obtained from malaria-treated serum.

One of Noguchi's cultures, " McD.," and Zinsser's culture were kindly given

by the Inoculation Department, St. Mary's Hospital, W., and on sub-cultures from these all the investigations have been carried out.

Attention was first directed to a study of the agglutinating properties against the cultures that might be found in the various types of sera.

A liquid ascitic bouillon (1 part ascitic fluid, 2 parts broth), to which the spirochætes adapted themselves well, was used in the experiments. This broth medium, after growth had occurred, was spun for about 5 or 6 hours and the resulting deposit washed with normal saline and respun several times until the spirochætes were free from any detritus, such as kidney cells, etc. Saline was then added to the organisms until a suspension of such strength was obtained that when examined with dark ground illumination by 1/12 objective and 1/3 ocular, 20–25 organisms were visible in each field. The sera to be investigated were first inactivated and then diluted to the following strengths: 1/10, 1/20, 1/40, 1/80, 1/120, 1/160, etc., up to 1/400. 1 c.c. of each dilution was placed in a separate tube of the Dreyer type, to each of which was added 0·1 c.c. of the spirochætal emulsion which produced a faint grayish turbidity. Controls of 1·0 c.c. normal saline and 0·1 c.c. emulsion were put up for each serum investigated, both for ease of reading and as an attempt to control the controls. The sera were then incubated in a water-bath at 37° C. for 2 hours when the first reading was made, they were then left at room temperature for 18–24 hours when the second reading was taken. The agglutinations were read macroscopically and with a hand lens. The flocculation which occurs in positive readings is very fine, rather resembling that found in dysentery agglutinations and the end-point is very indefinite. The whole reaction lacks the clear-cut results or definition which may be obtained in typhoid agglutinations, etc. The 24-hour readings were the more stable since there is a tendency for further agglutination to occur after the tubes have been taken out of the water-bath, but which ceases in about 12 hours.

THE AGGLUTINATING PROPERTIES OF SERA.

The total number of sera examined was 160. 110 of these were without malaria treatment, 50 being from non-syphilitic psychotics whose blood gave a negative Wassermann reaction, and the remaining 60 from syphiltic psychotics though not all were suffering from syphilis of the central nervous system. The remaining 50 sera were obtained from paretics who had received malaria treatment. The large majority of these treated cases, over 90%, had made such a good clinical recovery, as also was the case in the treated sera referred to in other experiments, as to justify their discharge from a mental hospital. The few remaining treated patients from whom sera were obtained had recovered from the acute stage, but were still under institutional care.

An analysis of the results obtained from untreated sera with a negative Wassermann reaction shows that 30% give no evidence of possessing agglutinin in any dilution; the highest titre of agglutinin, this only occurred in 10% of cases, was found in dilutions of 1 in 120, whilst 60% of the sera agglutinated the antigen in a dilution of 1 in 20. Whereas all sera with a positive Wassermann reaction agglutinated the organisms in a dilution of 1 in 40, 60% give a definite agglutination in dilutions as high as 1 in 120, a further 20% giving a

plus-minus reaction in the same dilution. Sera from cases who have received malaria treatment showed a general tendency to possess a higher agglutinin content than either of the foregoing, irrespective of the Wassermann findings. One serum agglutinated the organism when diluted to 1 in 280, 30% in a dilution of 1 in 240—one of these having a negative Wassermann reaction—and 50% in dilutions of 1 in 200. 8% exhibited the Neisser Wechsberg pro-agglutinoid phenomenon; although the percentage is small this is remarkable in that all the sera showing this type of agglutination were obtained from patients undergoing a subsidiary course of treatment with tryparsamide. Twelve cerebro-spinal fluids were put up against the cultures; unfortunately they all possessed a positive Wassermann reaction though 4 were from malaria treated cases. The titres of agglutinin were considerably less than those obtained with the corresponding sera, but the treated fluids showed the same tendency to agglutinate the spirochæte in higher dilutions than non-treated fluids.

SPIROCHÆTICIDAL PROPERTIES OF SERA.

Attention was next turned to the investigation of the treponemicidal properties, if any, of the various types of sera, that is, sera obtained from untreated patients possessing either a negative or positive Wassermann reaction and sera from patients who had been treated by induced malaria.

Graduated quantities of such sera ranging from 0·3 c.c. to 0·005 c.c. were put up with equivalent amounts of fresh complement, 0·1 c.c. of spirochætal culture medium, containing about 40 live active organisms per field when examined under dark ground illumination, added to each tube and all were brought up to total volume of 1·1 c.c. with the appropriate amounts of normal saline. These tubes were then plugged and incubated in a water-bath at 37° C. for 2 hours when 1·0 c.c. from each was withdrawn and added to tubes containing 3·5 c.c. standard culture medium, fresh rabbit kidney having been previously added. These second tubes were covered with paraffin, sealed and incubated for 21 days when they were examined for evidence of growth, number of organisms per field, motility, clumping, etc.

Of the sera examined the results of only 21 treated cases and 28 untreated cases can be considered, the others having been contaminated by various organisms. All cultures which were apparently sterile were sub-cultured with consistently negative results, no further growth occurring.

For simplicity only the results obtained with 0·1 c.c. of serum need be considered since it was found by experience that in no case did untreated sera cause any obvious alteration in growth with this quantity. That such sera produced no effect on the growth of the organism was to be expected for one had previously found that the spirochæte flourished equally as well in ascitic fluid obtained from either a syphilitic or non-syphilitic patient.

At first the results obtained with treated sera were conflicting, some causing complete inhibition of growth, whilst others in quantities up to 0·3 c.c. had no apparent detrimental effect on the micro-organism. When, however, sera were arranged in order of the length of time that had elapsed since they were collected and the cessation of malaria the cause of this inconsistency was clear. In tubes to which had been added serum collected not more than 5 months

after the termination of malaria there was complete inhibition of growth. It was likely that such suppression occurred in serum up till 12 months after malaria, for the few organisms that were found in tubes containing sera up to this period were non-motile, degenerate, and might possibly be the original organisms which had been added to the sera. From 12 months up to 18 there was a tendency for the spirochætes to be more active and more numerous per field until after 18 months malaria treated serum showed no cultural differences from ordinary serum. Such findings seemed to strengthen the belief that malaria caused in the host the production of some body, or bodies, inimical to the growth of cultured spirochætes. It is possible that the possession by these sera of such a property was a coincident and in no way due to the previously induced malaria. Obviously one had to follow patients throughout their malarial history, from the incubation period to well beyond the cessation of rigors, in order to eliminate this possible fallacy. If any alteration occurred in the serum of such patients one was more justified in assuming that such a change was directly due to malaria.

Six patients were followed out from the incubation period till 2 weeks after cessation of the rigors. During the incubation period no sera produced any diminution of the number of spirochætes per field (about 50) and all were actively motile, this continued until the fourth rigor when motility became diminished in 2 of the sera ; at the sixth rigor no growth occurred in 2 tubes, whilst 2 others had only 1–4 non-motile degenerate organisms per field ; at the tenth rigor only 1 tube showed any organisms and in this case only 5 non-motile spirochætes per field ; 2 weeks after the serum from the same patient still showed a few non-motile organisms, the other tubes all being sterile.

CONCLUSIONS.

1. Many sera with a negative Wassermann reaction are able to agglutinate cultured treponemata in the lower dilutions.
2. Sera which give a positive agglutination in dilutions of 1 in 80 or more are almost certainly syphilitic.
3. Malarial treated sera and cerebro-spinal fluids show a higher agglutinin content than those not so treated.
4. Since such a relatively large percentage of Wassermann negative sera give a positive agglutination reading in the lower dilutions any increase in agglutinin is not necessarily specific.
5. The agglutination test is of no value in the diagnosis of syphilis owing to difficulties of technique and lack of definition.
6. Untreated sera obtained from either syphilitics or non-syphilitics give no evidence of possessing treponemicidal properties to the cultures used.
7. Whereas malarial treated sera do so, their spirochæticidal strength depends on the time that has elapsed since the termination of the malaria.

In conclusion I would express my indebtedness to Dr. Golla and the Central Laboratory staff for their helpful suggestions and cooperation in this work ; also to the Inoculation Department, St. Mary's Hospital, Paddington, W., for the cultures and useful advice they so kindly gave me.

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THE FAVOURABLE INFLUENCE OF GELATIN IN THE MEDIUM ON THE ACTIVITY OF A STAPHYLOCOCCUS BACTERIOPHAGE.*

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IT is well known that addition of a fairly high concentration of gelatin inhibits and may even prevent the clearing of a susceptible culture by the bacteriophage. Yet, in working with the staphylococcus bacteriophage "B.H." secured through the courtesy of Dr. André Gratia of Brussels, the addition of 5% gelatin to bouillon was found to accelerate both the clearing of the culture and the multiplication of the bacteriophage. The following experiments demonstrate the effect clearly.

The original bacteriophage "B.H." as received from Dr. Gratia was titrated by serial dilution in two sets of tubes. In one set each test-tube contained 5 c.c. broth. In the other each tube contained 5 c.c. broth to which 5% gelatin had been added. The dilution was made 1 in 100 each time by transferring 0·05 c.c. from each tube to the next succeeding one. The gelatin tubes received their 0·05 c.c. from the broths of the preceding dilution, so that the two series were perfectly comparable whatever be the error of dilution. The time (in hours) taken by each tube to clear at 37°C. is mentioned under the degree of dilution in Table I.

TABLE I.

	10^{-4}.	10^{-6}.	10^{-8}.	10^{-10}.	10^{-12}.
Titration in broth .	>24 hours .	48 hours .	48 hours .	∞ .	∞
,, in gelatin-broth .	24 ,, .	24 ,, .	48 ,, .	∞ .	∞

After 24 hours, the 10^{-4} dilution tubes were filtered and the amount of bacteriophage that had grown in each was estimated. At this time the

* Part of a thesis approved by the University of London for the Ph.D. degree.

broth-tube was turbid while the gelatin-broth tube was clear. The result, as found next day, is shown in Table II.

TABLE II.

	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.	10^{-9}.	10^{-10}.	10^{-11}.	10^{-12}.
Filtrate of broth 10^{-4}	+	+	+	+	+	−	−	−	−
„ of gelatin-broth 10^{-4}	+	+	+	+	+	+	+	−	−

The bacteriophage had multiplied to a greater extent in gelatin.

After 48 hours, the 10^{-6} dilution tubes of the original series were filtered and the amount of bacteriophage that had grown in each was estimated. At this time both the tubes were clear, but while the broth tube had just cleared, the gelatin-broth one had been standing clear for at least 24 hours. The result was as follows:

	10^{-4}.	10^{-6}.	10^{-8}.	10^{-10}.	10^{-12}.
Filtrate of broth 10^{-6}	+	+	+	−	−
„ of gelatin-broth 10^{-6}	+	+	−	−	−

The bacteriophage in the gelatin-broth tube had gone to its full titre and already started diminishing.

This experiment was repeated with bacteriophage "B.H." that had undergone a few passages through the susceptible staphylococcus. The result was practically the same.

The clearing effect of this latter bacteriophage as well as of the original "B.H." on a different staphylococcus was tried. Once again clearing occurred much more rapidly in the 5% gelatin-broth than in plain broth.

An experiment was then performed to find out the optimum concentration of gelatin for bacteriophagy. Gelatin was added to broth tubes to make varying concentrations from 0·1% to 10%. To each tube of one such set was added ½ c.c. of bacteriophage diluted 1 in 10^2. To another was added ½ c.c. of the same bacteriophage but diluted 1 in 10^4. The third set received no bacteriophage. All the tubes were planted with the same amount of staphylococcus and incubated. Next day it was found that the tubes of the first two series containing 5% and 2·5% gelatin were clearer than the rest. These were also the first tubes to clear completely. That the gelatin had no inhibiting action on the growth of the staphylococcus was shown by the profuse turbidity produced in all the tubes of the third set.

Precaution was taken to ensure that the medium in all the tubes had a pH of 7·8. On the addition of gelatin to broth the hydrogen-ion concentration gradually rises until after about 24 hours the reaction is very acid. This is due to the slow precipitation of alkaline phosphates. In the present experiments the medium was pipetted off after precipitation and brought to the correct pH before use. It may be remarked, however, that even in the less alkaline 5% gelatin medium bacteriophagy occurred more quickly and was more complete than in plain broth at pH 7·8.

Further investigation with other races of the staphylococcus bacteriophage, with other bacteriophages and with other organisms has not yet been completed. It is possible the favourable effect is restricted to the staphylococcus

and other gelatin-liquefying organisms. In any case the results of these experiments show that viscosity is not the only factor acting in the influence of gelatin on the course of lysis.

CONCLUSION.

A low concentration of gelatin (about 5%) in the medium has been found to accelerate the action and multiplication of a staphylococcus bacteriophage.

THE *RÔLE* OF OXYGEN IN BACTERIOPHAGY.*

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IT is not generally realized that the depth of fluid in which a bacteriophage is made to act has a marked influence on the rate and extent of lysis. Little attention is paid, as a rule, to the size of test-tubes used in experiments on the bacteriophage, and though slight variations in diameter would probably have no effect on the course of lysis, yet the inadvertent use of a flask or a very wide tube in place of the ordinary test-tube may make considerable difference in the result of an experiment. Indeed, it was the occasional finding of aberrant results in the course of other work that drew the writer's attention to the possibility of such an effect.

Shwartzman (1925) has reported some experiments, the results of which led him to believe that a restriction of air supply was necessary for the action and growth of the bacteriophage. Working with a bacteriophage that was active in 5 c.c. of a dilution of 10^{-8}, he made a dilution of 10^{-9}, and distributed equal quantities (varying from 1 to 5 c.c.) in test-tubes of different diameters. In the narrow tubes the culture was dissolved and the bacteriophage grew to its original titre in 24 hours. In the wide tubes lysis was absent and the bacteriophage could not be recovered next day. In his protocols there is no evidence that the non-lysed tubes had any bacteriophage at all to start with. In such an ultra-terminal dilution as he used the presence of bacteriophage in any individual test-tube would be entirely fortuitous. Indeed, the evidence is strongly against the initial presence of bacteriophage in the tubes that did not clear, since the bacteriophage could not be recovered from any of them. The results are so clean-cut, lysis and full regeneration, or no lysis and no regeneration, that one is forced to the conclusion that the non-clearing tubes had no bacteriophage delivered into them.

* Part of a thesis approved by the University of London for the Ph.D. degree.

Later, d'Herelle (1927) investigated the *rôle* of oxygen in bacteriophagy, and came to the conclusion that exposure of a large surface to air hastens and promotes the action of the bacteriophage. This is in agreement with the rapid action of the bacteriophage on solid media and the writer's results also completely bear out the view.

In the course of other work with the bacteriophage the writer had noticed the occasional persistence of turbidity in an intermediate tube of a dilution series, while tubes on either side of it cleared perfectly well—the so-called zonal effect. In the ignorance of the work of Shwartzman and d'Herelle, no attempt had been made to use test-tubes of uniform diameter and these varied from 12 to 19 mm. There was a vague impression left in the author's mind that the offending tube in these zonal phenomena was usually one of narrow diameter. This suspicion gained strength from a chance observation that in a wide tube (18 mm. diam.) containing only 2·5 c.c. of a particular bacterium-bacteriophage mixture, profuse secondary growth occurred as early as in 24 hours. The following investigation was therefore undertaken in order to find out if any relationship existed between the depth of fluid and the course of lysis.

A coli-bacteriophage was used. It was active in 5 c.c. of a dilution of 10^{-11}. A dilution of 10^{-9} was made in broth (pH = 7·8), and 200 c.c. of it was lightly inoculated with the susceptible organism. The mixture was then distributed with a graduated pipette according to the scheme in Table I. It was thus contrived to obtain (1) different depths of fluid for the same surface and (2) different depths for the same volume.

The results are given in Table I. The ratio $\frac{\text{area of surface}}{\text{volume}}$ (*i. e.* the reciprocal of the effective depth) is usually taken as the measure of availability of oxygen in a fluid. To bring out clearly the relationship between the rate of lysis and the different values of this ratio the table is re-arranged in the order of these values. The delay in lysis accompanying increase in the effective depth is quite plain.

The effect was investigated in the case of the staphylococcus bacteriophage in a different way. In each of 12 test-tubes of 17 to 19 mm. diameter were placed 10 c.c. broth (pH = 7·8) and 0·0005 c.c. bacteriophage. The tubes were then lightly and equally sown with the susceptible staphylococcus. In the incubator 6 tubes were stood upright and 6 were sloped so as to have a maximal surface. Controls were kept, both upright and slanting, without bacteriophage.

The results are given in Table II. The sloped tubes cleared quicker than the upright ones. The sloped control showed more profuse growth (greater turbidity and acidity) after 24 hours than the upright one, showing that the free supply of air promoted bacterial growth also.

In another paper the author (1930) has described the favourable effect of a low concentration of gelatin on the action of this bacteriophage. To determine if free exposure to air would add to this favourable effect, the experiment was repeated in this enriched medium (broth containing 5% gelatin, pH = 7·8). Once again the sloped tubes cleared quicker and the slanting control showed more profuse growth and, in either case, the clearing was quicker than in the corresponding broth preparations (Table III).

TABLE I.—*Progress of Lysis in Varying Depths of Lytic Mixture.*

Diameter of test-tube in mm.	Volume of lytic mixture in c.c.	Area of surface / Volume.	Degree of lysis after 24 hours.	Degree of lysis after 48 hours.	Degree of lysis after 4 days.
13	2·5	0·53	+	+	0
	5	0·26	0	+	0
18	2·5	1·0	+	±	0
	5	0·50	±	+	0
	10	0·25	0	0	±
25	2·5	1·88	+	±	0
	5	0·94	±	+	0
	10	0·47	0	0	±
	20	0·24	0	0	±

+ = complete lysis; ± = partial lysis; 0 = no lysis (turbid).

By the word "lysis" is meant visible clearing, irrespective of any true lysis tha tmay have been obscured by rapid bacterial growth. The turbidity following "lysis" is due to secondary growth.

Each tube was made in triplicate, so that altogether there were 27 tubes. The three samples behaved exactly alike in each case.

The table rearranged according to surface/volume shows:

Surface (in sq. cm.) / Volume (in c.c.).	Degree of lysis after 24 hours.	Degree of lysis after 48 hours.	Degree of lysis after 4 days.
1·88	+	±	0
1·0	+	±	0
0·94	±	+	0
0·53	+	+	0
0·5	±	+	0
0·47	0	0	±
0·26	0	+	0
0·25	0	0	±
0·24	0	0	±

TABLE II.—*The Course of Lysis in Upright and Slanting Broth-Tubes.*

	Upright.	Slanting.
After 24 hours	0 0 0 0 0 0	+ + + + 0 0
„ 48 „	0 0 0 0 0 0	+ + + + + +
„ 72 „	+ + + + 0 0	

+ = lysis; 0 = no lysis.

Each + or 0 represents a test-tube (twelve in all). The presence of bacteriophage in every test-tube was confirmed by making agar subcultures.

TABLE III.—*The Course of Lysis in Upright and Slanting Gelatin-Broth-Tubes.*

	Upright.	Slanting.
After 24 hours	0 0 + + + +	+ + + + + +
„ 48 „	0 0 + + + +	+ + + + + +
„ 72 „	+ + + + + +	

See note under Table II.

The experiments described in Tables II and III were performed at the same time and with the same relative amounts of bacteria and bacteriophage.

It is clear, then, that lysis takes place more readily in shallow cultures than in deep ones. It follows that in quantitative work with the bacteriophage, the test-tubes used must be of a suitable and uniform diameter.

CONCLUSION.

D'Herelle's finding that lysis is accelerated by free exposure of the fluid to air is confirmed.

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IMMUNIZATION OF CHICKENS AGAINST FOWL-POX WITH DEAD, FORMOLIZED AND PHENOLIZED VIRUS.

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THERE are many investigations dealing with the question of immunization of fowls against fowl-pox with virulent, attenuated or dead virus. The results reported are variable and contradictory. Burnet (1906), Lipschutz (1913) and Manteufel (1910) failed to establish any degree of immunity with dead virus. Mack and Records (1916), and Fuller (1924) report favourable results with vaccine heated to 55° C. for one hour, while Boerner and Stubbs (1921) failed to establish any immunizing effect with heated virus. On the other hand Manteufel (1910) was able to immunize fowls with virus attentuated by treatment with bile; and Beach (1927) and others report favourable results with phenolized virus.

Apart from the practical aspect of the problem, it was of interest to determine whether living virus was or was not essential for immunization against fowl-pox. This question has a wider importance because of the different views of various investigators concerning the efficacy of dead virus in the immunization against virus diseases. Recent literature on the subject is replete with contradictory results. Todd (1928) and others have failed to immunize fowls against plague with heated material; Jouan and Staub (1920) report favourable results with formolized virus, while Doyle (1927) and Todd (1928) have been unable to confirm these findings. Similiar contradictory results are reported in connection with other virus diseases. Hunt and Falk (1927) obtained favourable results with vaccine virus killed by treating with weak solutions of formaline. Olitsky and Long (1928), however, have demonstrated

that formolized vaccines of stomatitis virus which produce immunity still contain living virus.

The virus of fowl-pox lends itself readily to experimental study of this problem. By using only the dry scab covering the lesions on comb and wattle, large quantities of virus can be obtained relatively free from protective proteins, which are very probably responsible for the contradictory results reported in the literature. This virus was therefore employed to determine on the one hand the lethal effects of heat, formaline and phenol on the virus, and on the other the immunizing effect of virus treated by the various measures indicated.

EFFECT OF HEAT, FORMALINE AND PHENOL ON FOWL-POX VIRUS.

Vaccine A.—An emulsion of fowl-pox virus was prepared by triturating 1·0 gm. of the scab in 10 c.c. Locke's solution. The material was thoroughly ground with the aid of sterile sand and the suspension centrifugalized at 1500 revolutions for 10 minutes. The supernatant fluid was then diluted with an equal volume of Locke's solution, giving a final dilution of 1/20.

This material was divided into three parts: One part was diluted with an equal volume of Locke's solution and heated to 56° C. for one hour. The second portion was mixed with an equal amount of 1% commercial formaline in Locke's solution. The third portion was mixed with an equal portion of 0·5% phenol in Locke's solution.

The three mixtures were kept for 4 days at room temperature and then tested. Three series of chickens were given 0·2 c.c. of the respective vaccine subcutaneously and about half that amount was scarified on the comb. The heated and formolized vaccines produced no effect, whereas the phenol-treated vaccine produced a typical lesion on the comb only slightly less extensive than that in the controls inoculated with glycerinated material.

One month after the vaccination the chickens were tested for immunity with fresh virus. These tests showed that the fowls treated with phenolized vaccine which still contained living virus were immune, while those treated with heated or formolized virus which had failed to produce lesions possessed no immunity.

This experiment was repeated with the same results. It appears, therefore, that heating the diluted virus suspension at 56° C. for 1 hour or treating it with 0·5% formaline for 4 days kills the virus and destroys its immunizing property. The negative results obtained with heated and formolized vaccine did not warrant further repetition of these tests. Subsequent experiments were therefore designed to determine the length of time phenolized vaccine still retained its virulence and the immunizing properties of the avirulent virus.

EFFECT OF PHENOL SOLUTION ON FOWL-POX VIRUS.

Vaccine C.—0·5 gm. dry scab taken from the comb of an infected chicken was ground with a small amount of sand and 5·0 c.c. Locke's solution added slowly. The suspension was allowed to stand until the heavy particles sedimented. The supernatant fluid was removed and the sediment washed with 5·0 c.c. Locke's solution. The virus suspension together with the 5·0 c.c. sand

washings were mixed and placed overnight in an ice-box. The next day the virus suspension was mixed with an equal volume of 0·5% phenol solution. The final vaccine was thus a 1/40 dilution of the original material in 0·25% phenol in Locke's solution. This vaccine was placed in the ice-box and tested at various intervals.

Tests.—1. Immediately after its preparation a drop of the vaccine was rubbed on a scarified wattle. A typical lesion appeared after 5 days and progressed normally.

2. After 8 days in the ice-box the vaccine was re-tested, as above, on the comb of a fresh chicken. After 7 days one vesicle appeared at the point of inoculation.

3. After 20 days 5 chickens were vaccinated, 0·2 c.c. being injected subcutaneously on the leg, and a smaller amount rubbed on a scarified surface. Two of the chickens showed slight lesions; one was doubtful, and the remaining two were negative. These chickens were re-tested with active virus after 52 days. In all slight lesions developed after 4 days and disappeared 5 days later. The prompt appearance of the lesions, their mild character and rapid disappearance indicate the presence of a definite immunity.

Vaccine D.—This vaccine was prepared as above from scabs on the comb of an infected chicken, 0·6 gm. virus being ground with a small amount of sand in 6·0 c.c. Locke's solution. The suspension was mixed with an equal amount of 0·5% phenol solution in saline and kept at room temperature. This vaccine differed from the previous one in being more concentrated; the final dilution was 1 : 10 instead of 1 : 40.

After 25 days 1 chicken was given 0·2 c.c. of the concentrated material subcutaneously on the thigh, and 0·1 c.c. was rubbed on the scarified wattle. Another set of 2 chickens were given the same virus diluted 1/4 in the same manner as above. The first chicken developed an active infection with metastasis on the tongue which subsided only after a month. Those chickens which received the diluted vaccines developed small lesions on the inoculated wattle after an incubation period of 10 days, and the lesions disappeared 2 weeks later.

After 35 days the vaccine was divided into 2 parts. One part was heated to 56° C. for 1 hour, while the second part remained unheated. One chicken received 0·2 c.c. of the heated phenol vaccine subcutaneously on the leg and 0·1 c.c. was scarified on the wattle. A second chicken was treated as above with unheated vaccine. Neither of the chickens developed lesions. After 15 days both were infected with fresh virus. Chicken 1 developed a typical lesion after an incubation period of 7 days. Chicken 2 developed a scab at the point of the inoculation after 3 days, but the scab dropped 2 days later and the lesion was healed on the fifth day.

Subsequent tests with the 50-day-old unheated vaccine gave a mild infection after the inoculation of a massive dose on the scarified wattle.

IMMUNIZATION WITH PHENOLIZED VACCINE.

In the course of these experiments over 30 chickens have been vaccinated with phenolized vaccine. Ir all cases where this vaccine was used at the time when it still occasionally produced a mild lesion, one dose sufficed to produce

immunity. The maximum duration of the immunity has not been ascertained, but in one series the chickens were immune when tested 2 months after the vaccination. A characteristic feature of the inoculation of immune chickens was the early appearance of transient lesions; these lesions appeared 2 to 4 days after the inoculation and healed with equal promptness. In one case a massive inoculation involving the entire comb resulted in the appearance 4 days later of 6 small vesicles, which disappeared in another 5 days.

It appears, therefore, that the vaccination of fowls with phenolized vaccine affords a considerable degree of protection. The important point, apparently, is to test the vaccine regularly and use only material which still contains live virus, sufficiently attenuated not to produce active lesions. This stage cannot be predicted, since it depends on the method of preparation and on the concentration of the vaccine, and most probably also on the concentration of the proteins in the suspension. The different results obtained with vaccines C and D respectively are illustrative. The first vaccine, which was diluted 1 : 40, no longer produced lesions after 20 days, while the second, which was more concentrated, still produced active lesions after 25 days, and these lesions were more severe when the concentrated vaccine was used than when it was diluted to 1 : 4. This latter vaccine still produced a mild lesion in a susceptible chicken after 50 days. The lethal effect of phenol on fowl-pox virus is evidently much slower than that of formaline and the rate of attenuation seems to depend on the concentration of the virus suspension.

CONCLUSIONS.

1. Fowl-pox virus heated to 56° C. for 1 hour or treated with a 0·5% formaline solution for 4 days no longer produces lesions in susceptible chickens and also fails to induce immunity.

2. Phenolized vaccine (0·25% phenol) produced from the scab of the lesion still contains active virus 20, 25 and 50 days after the preparation, the survival of the virulence depending on the concentration of the vaccine suspension.

3. One injection of a phenolized vaccine which no longer produces active lesions is sufficient to produce immunity.

4. The failure of heated phenolized vaccine to produce immunity and the success obtained with the same material unheated indicates that the immunity was induced by the surviving live virus.

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SEROLOGICAL STUDIES IN THE GROUP OF THE SPORE-BEARING ANAËROBES. II. *IN VIVO* EXPERIMENTS WITH AN "O" IMMUNE SERUM TO *VIBRION SEPTIQUE* DEVOID OF ANTITOXIN CONTENT.

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In a previous paper (Felix and Robertson, 1928), it was demonstrated that the spore-bearing anaërobes as exemplified by *B. œdematis maligni* and *B. tetani* showed the two types of antigen known as the "O" and the "H." It was also stated that only the immune body corresponding to the "O" antigen was active in complement-fixation.

In the present paper the results are given of an inquiry into the behaviour of this immune body in protection against infection with *B. œdematis maligni* (*Vibrion septique*). In *B. typhosus* and the Salmonella group of organisms evidence has been adduced suggesting that the antibody corresponding to the "O" antigen plays a predominating part in immunity to infection (Felix, 1924; Felix and Olitzki, 1926; Arkwright, 1927; Ibrahim and Schütze, 1928; Lubinski, 1928; Topley, 1929). The interest of this study in the case of the anaërobes is enhanced by the fact that a soluble toxin whose action is well recognized is also produced and comparative data could be secured by the parallel use of antitoxic sera.

The work was made possible by the kind co-operation of our colleague Dr. Petrie, who undertook the task of immunizing the horses at the Lister Institute farm at Elstree. We relied on his practical experience in obtaining the immune serum and we are glad of this opportunity of thanking him. Our thanks are also due to Dr. O Brien of the Wellcome Research Laboratories for a valuable supply of monovalent antitoxic serum to *Vibrion septique*. We are further indebted to Dr. Weinberg of the Pasteur Institute for samples of various antisera.

(1) *The "O" Immune Serum Used.*

The "O" immune serum was made by the intravenous injection into a horse of washed *Vibrion septique* bacilli belonging to the strain "Pasteur III" (Group I of Robertson, 1920), heated to 100° C. for two hours. The exact method of preparing the antigen is given in the appendix. Considerable care has to be exercised in the immunization, and this particular horse finally succumbed apparently through protein shock. The danger of this reaction to the doses of vaccine was, however, overcome in the case of other immunizations with *B. tetani* and *B. welchii* at present in progress.

A prolonged course of injections with very large quantities of vaccine are necessary to produce a serum of effective action. The titre of the "O" immune serum used in this work was determined by the death of the horse which occurred after the intravenous administration of an amount of vaccine corresponding to the growth of 32 litres of broth culture; it therefore gives no information about the upper limit of its effectiveness. This investigation is merely the study of a given serum of an adequate titre. The serum was used unconcentrated and without preservative, and, kept at a low temperature in the dark, it retained its quality during at least nine months.

As the animal was inoculated with bacilli heated to 100° C. it was not expected that the serum would contain any antitoxin at all, but the point was nevertheless tested and no protection whatsoever was shown in rabbits against intravenous injection of the toxin. The serum was also tested with negative results for the presence of "H" agglutinins. It showed "O" agglutinins and was also capable of binding complement; the *in vitro* analysis of this and other immune sera to *Vibrion septique* will be given in another communication.

(2) *Method of Experiment.*

It is now clearly understood that with spore-bearing anaërobes the nearest approach to the natural infection is obtained by the use of washed spores (freed from toxin) injected along with some kind of an activator. This method has been used by a number of workers; Bullock and Cramer (1919), Tenbroeck and Bauer (1926), Fildes (1927) and Russell (1927) all give details of their procedure, and it is now a well-established technique. After some preliminary attempts in various directions the following type of experiment was found to meet the requirements and was used throughout. The cardinal points are (*a*) an absolute certainty of infection, and (*b*) some indication of the relative severity of the test presented.

We came to the conclusion that absolute standardization of the infecting dose was impracticable, but that a very good analysis could be made by including in each experiment a titration of the dose used. The activator employed was, in the great majority of cases, calcium chloride, and a 3% solution was found to be a quite certain activator of washed spores when used in a dose of 0·25 c.c. for mice. Calcium chloride is not an entirely innocuous substance for mice, and this dose cannot be much increased without a definite danger of calcium chloride deaths, so that if sickly individuals are included a certain small number of adventitious accidental deaths from other causes may occur. On the other hand, lesser doses gave an irregular number of failures to activate the spores and limited the downward titration of the infecting dose. Swellings and even small perforating necrotic lesions due to the calcium may also arise. It is advisable to use freshly distilled water for the $CaCl_2$ solutions, which are of course sterilized.

In the earliest experiments the serum and the infecting dose were given simultaneously, but this was found to complicate the technique with no corresponding advantage, and in the great bulk of the work (including all that done with the horse-serum), the mice received the serum two days before the injection of the spore-calcium suspension.

All injections were made intra-muscularly; in the animals treated with serum the latter was injected into one leg, and the infecting mixture two days later into the other.

In a typical experiment the scheme comprised:

I. Control without spores, the mice receiving the activator alone in the dose used.

II. Control without $CaCl_2$, the mice receiving only the washed spores in the dose or in twice the dose of the highest opacity used.

III. Titration of the infecting dose—that is, control of spores with $CaCl_2$ without serum. The spores suspended in saline were matched with opacity No. II of Burroughs, Wellcome & Co.'s series. The spores were used in this opacity as the maximum, and also in dilutions in saline of 1/100, 1/1000, 1/10,000 and 1/100,000. The infecting suspension contained 0·25 c.c. of the spores in the various dilutions in saline, with 0·25 c.c. of calcium chloride (3% solution) and 0·2 c.c. of saline, the total volume being 0·7 c.c.

IV. Serum controls with normal sera; the mice received 1 c.c. of the serum two days previously, and were then injected with usually the first two concentrations, that is opacity II, and opacity II diluted 1/100, along with the standard dose of the activator. Sometimes a group with a third concentration was included.

V. Animals injected with the immune sera to be tested. The animals received 1 c.c. of the immune sera two days previously; they were then injected with the spore-calcium suspension in exactly the same way as in IV, titrated to the particular point selected.

VI. Calcium controls of all the serum groups included in the experiment. That is, two mice from each group of the animals which had received the serum were injected with 0·25 c.c. of the 3% calcium chloride solution to test for the toxicity of the calcium in animals treated with serum.

In the downward titration of the spores in III there is no implication of multiple lethal or infecting doses; all that it conveys is an indication of the relative severity of the test, and it can be used as a means of comparison between different experiments. In general the lower dilutions of the spore suspension always produced death of all the mice in 16 to 24 hours in the titration without serum (III of the above scheme), and in the normal serum controls (IV of the scheme). In the highest dilutions irregular deaths or survivals indicated that the number of viable spores was below the certainly infecting level.

The most frequent source of error in obtaining the correct balance in these experiments was the failure to free the spores from toxin. (See appendix for the exact preparation of the spore suspension.)

(3) *The Effect of the Sera Tested.*

Table I gives a sample experiment (No. 32 abridged), in which the bacterial suspension chosen was opacity II diluted 1/100 with saline, the activator was 0·25 c.c. of a 3% solution of calcium chloride, the total volume made up to

TABLE I.

No.	Previous treatment 4/3/29.	Material injected 6/3/29.	Total volume.	Number of mice.	Result.
I	*Nil* ($CaCl_2$ control)	0·25 c.c. of $CaCl_2$ 3% solution	0·7 c.c.	4	All survived without symptoms.
II	„ (spore control)	0·25 c.c. spores, Pasteur III, opacity II	0·7 „	4	„ „
III	*Nil* (titration of the infecting dose)	$CaCl_2$ 3% solution with Pasteur spores, opacity II	0·7 „	2	All dead with gas gangrene within 16 hours
		$CaCl_2$ 3% solution with Pasteur spores, opacity II/100	0·7 „	4	All dead within 16 hours.
		$CaCl_2$ 3% solution with Pasteur spores, opacity II/1000	0·7 „	3	„ „ 18 „
		$CaCl_2$ 3% solution with Pasteur spores, opacity II/10,000	0·7 „	2	1 dead in 18 hours. 1 „ 22 „
IV	1 c.c. normal horse-serum "Vibrio I"	$CaCl_2$ 3% solution with Pasteur spores, opacity II/100	0·7 „	6	5 „ 18 „ 1 „ 22 „
V	1 c.c. GG 2639A *Vibrion septique* antitoxic serum	Ditto	0·7 „	10	4 „ 24–30 hours. 2 „ 42 „ 2 „ 66 „ 1 „ 110 „ 1 survived with considerable local lesion which healed well.
	1 c.c. anti-*Vibrion septique* serum 329	„	0·7 „	10	5 dead in 24–30 hours. 3 „ 42–44 „ 2 survived with slight local lesions.
	1 c.c. "O" immune serum to *Vibrion septique* devoid of antitoxin "Vibrio IV"	„	0·7 „	10	All survived with local lesions which healed completely, the most severe with slight contraction of the leg injected.
VI	1 c.c. serum "Vibrio I"	0·25 $CaCl_2$ 3% solution	0·7 „	2	All survived without symptoms.
	1 „ „ GG 2639A	„ „	0·7 „	2	(These are the controls for the
	1 „ „ 329	„ „	0·7 „	2	toxicity of the $CaCl_2$ solution in
	1 „ „ "Vibrio IV"	„ „	0·7 „	2	animals injected with serum.)

0·7 c.c. with saline. The infecting capacity of the mixture was such that opacity II diluted to 1/10,000 in the same combination with calcium produced death in 18 to 22 hours; the end-point at which infection no longer occurred was not obtained in this particular experiment.

The high-power antitoxic serum GG 2639A and the serum 329 which was stated to be antibacterial as well as antitoxic to *Vibrion septique* were only able to protect a very small proportion of the mice, while the "Vibrio IV" serum, which is the "O" immune serum devoid of antitoxin, was able to prevent the death of all the animals. The antibacterial content of the three immune horse-sera as demonstrated by agglutination and complement-fixation tests with the strain "Pasteur III" is shown in Table II:

TABLE II.

Serum.	Antigen Pasteur III.		
	Agglutination.		Complement-fixation titre.
	"H"-titre.	"O"-titre.	
GG 2639A	<1 : 50	<1 : 50	<1 : 100
329	1 : 100,000	<1 : 50	<1 : 100
"Vibrio IV"	<1 : 50	1 : 500	1 : 1000

Serum GG 2639A = monovalent antitoxic serum to *Vibrion septique*, Group II. Horse immunized with filtered toxin (from Dr. O'Brien).

Serum 329 = antitoxic and antibacterial serum to *Vibrion septique*. Horse immunized with formolized total culture ("anaculture") (from Dr. Weinberg, Paris).

Serum "Vibrio IV" = monovalent antibacterial serum to *Vibrion septique*, Group I. Horse immunized with washed bacilli heated to 100° C. for two hours.

It must be observed that this antibacterial "O" serum was found to have a high protective power in the saving of life, but (in this form of experiment with calcium chloride) a certain degree of local swelling or even of perforating gangrene occurred from which the animals recovered completely. If the infectivity of the dose was very high, and the gangrene relatively severe, there might be some sloughing and subsequent contraction on healing. This reduction of a fulminating gangrene fatal within 24 hours to a restricted local lesion of varying, but often of quite unimportant dimensions, was most striking, and was the invariable effect of the "O" immune serum when injected previous to a spore-calcium suspension of "Pasteur III"—that is, of the homologous strain.

Of 40 mice which were at different times protected by 1 c.c. of this serum, 3 died from causes probably due to the local gas gangrene, after periods of from 78 to 164 hours, while a fourth died from an intercurrent coccal infection after 90 hours. Of the first 28 of the mice treated in the above group with 1 c.c. of the "O" immune serum 2 died, and in an exactly parallel set of 28 injected with 1 c.c. of the antitoxic serum GG 2639A, 13 died of gas gangrene. In these experiments where spores were injected with calcium as activator, the antitoxic serum had a much lower life-saving power than the "O" immune serum. In certain experiments, however, where a downward titration of the spore suspension afforded a less severe test, a protective action

could, as stated above, be demonstrated by the pure antitoxic serum. In this case also, just as with the "O" immune serum, the fulminating gangrene was reduced to a local lesion—that is to say, the life of the animal was preserved, but a local gangrene was nevertheless set up. In a few cases a border-line was found where the lesion was negligible; this occurred with both sera, but in minimal attacks the antitoxic serum did seem to have some slight advantage. It must be borne in mind that in these experiments the necrosing action of the calcium chloride has to be made good, in addition to the specific attack of the bacillus.

The serum 329 was only used in one experiment (number 32 shown in Table I). It had apparently no action other than that afforded by its antitoxic capacity, which was considerable, as shown by the fact that its performance in Table I is rather better than that of GG 2639 A. The antibacterial content as tested *in vitro* was entirely of the "H" type (see Table II).

In cases where the bacterial attack was less severe, both the antitoxic serum GG 2639 A and the "O" immune serum could be used in the dose of 0·5 c.c. with effect, but it was interesting to find that a summation of the sera was useless unless an effective dose of each was present. There was in fact no summation of effect at this lower range of action. This result is in agreement with the fact that the mechanism of protection exerted by the two sera is widely different, the "O" immune serum having no neutralizing power against the toxin formed, while the antitoxic serum (GG 2639 A) would seem to have no direct action upon the bacteria.

(4) *Specificity of the Serum.*

After having demonstrated the protective action of the "O" immune serum against the homologous organism, experiments were made with *Vibrion septique* strains from other serological groups. The strain "Stokes" from Group II and the strain "Milk" from Group III were both used, and the serum "Vibrio IV" was quite unable to protect the mice, although death was delayed by a few hours in comparison with the controls. In experiments with Group II the antitoxic serum GG 2639 A was also tested, and it was found to have the same low degree of protection as with the Pasteur strain of Group I; that is to say, in so far as it had any protective action it was equally effective against Group I and Group II. It is itself a monovalent antitoxic serum made from a Group II strain. Its titre in "H" agglutination with the homologous (Group II) strain was 1/2000, but it was entirely inactive in "O" agglutination and complement-fixation; its reaction with strain "Pasteur III" (Group I) is given in Table II.

The "O" immune serum was finally tested against the strain "Amatzi" belonging to Group I. Protection was afforded in the same manner as against the homologous strain but to a slightly (though appreciably) less degree. It would appear, therefore, that the antibacterial "O" immune serum is specific to the serological group—a result that is in keeping with Tulloch's work (1919) on tetanus. Its bearing on the rather complicated work of Tenbroeck and Bauer (1926), who trace an extremely type-specific immunity in tetanus, is not yet clear.

The very high degree of specificity of the *in vivo* protective action of the "O" immune serum came as something of a surprise in view of the considerable overlapping of the "O" antigen established previously in agglutination and complement-fixation tests between the groups (Felix and Robertson, 1928).

One curious point deserves notice in conclusion, namely the ineffectiveness in mice of "O" immune sera made in rabbits of considerably higher titre (as judged by *in vitro* tests) than the highly protective horse-serum "Vibrio IV." The rabbit serum *in vivo* only prolonged the life of the mice, and in a few cases of slight infection it did actually protect, but the action was extremely feeble. The nature of the serum used for passive immunization in any particular species of animal does not appear to be negligible. Avery and Tillett (1929) have made a recent contribution to this subject. They found that the bacterial carbohydrates of pneumococcus produce rapid and fatal anaphylactic shock in guinea-pigs passively sensitized with anti-pneumococcus rabbit serum, while there is complete absence of anaphylactic response in guinea-pigs sensitized with anti-pneumococcus horse-serum.

(5) *Action of the "O" Immune Serum in Guinea-pigs Against Actively Proliferating Bacteria in Broth Culture.*

As a final experiment 4 guinea-pigs were injected intramuscularly with the antitoxin GG 2639 A, 4 with the normal horse-serum "Vibrio I" and 4 with the "O" immune serum "Vibrio IV." Two days later they were all inoculated in pairs with 1·4 c.c. and 0·8 c.c. of a 48-hour living broth culture of "Pasteur III" in glucose broth. As expected, the animals protected with the antitoxin survived without lesion, while those guinea-pigs which had the normal horse-serum were dead within 16 hours, and those injected with the "O" immune serum showed, in the animals which had received the higher dose of culture, severe gas gangrene, from which one died after three days while the other was chloroformed as the lesion was extensive; the two animals which had been given the lower dose (0·8 c.c.) survived with only a local gangrene of moderate severity which healed well. This is an indication of a powerful defence by the purely antibacterial immune body against a very rapid form of infection by means of actively proliferating bacilli and preformed toxin in a highly susceptible animal.

(6) *Discussion.*

The interest of the foregoing paper lies in the demonstration of a definite antibacterial action *in vivo* by the serum of a horse immunized with the "O" antigen only of a bacterial species which normally contains "H" antigen in addition and secretes a soluble toxin. We have shown here the potency of the "O" antibody in independent action against an organism with a high degree of invasive capacity. Here, as in the aerobes, a high titre of "H" antibody appears to be negligible in protection. Thus the enormous difference in "H" antibody between the serum GG 2639 A which had no "H" titre for strain "Pasteur III," and serum 329, which had 1/100,000 titre, finds no expression in the *in vivo* action. An antibacterial serum ("Vibrio IV") devoid of "H"

antibody and of antitoxin proved, on the other hand, to be definitely active. We conclude, therefore, that an antibacterial action *in vivo* cannot be inferred from a high titre in "H" antibody but can from the "O" titre.

We would add that the powerful antitoxic serum 329 of Dr. Weinberg (Weinberg and Barotte, 1929), while showing a very high "H" titre to Groups I, II and III, was devoid of any appreciable "O" titre to all four groups of *Vibrion septique*. Weinberg's consistent search for a gas gangrene serum both antitoxic and antibacterial in action is undoubtedly an attempt in the right direction. The importance of the addition of an antibacterial component to the antitoxic action is demonstrated in the foregoing paper. Weinberg's method of estimating this component by "H" agglutination is invalidated by the fact that it is only the "O" antigen and its corresponding immune body which are involved in antibacterial protective action.

We should like to point out in passing that experiments where spores are injected with an activator other than the toxin afford an excellent means of testing the antibacterial activity of a serum.

The antitoxic serum neutralizes toxin whether this is injected as a preformed substance or as a nascent product of bacterial growth within the body. This is its only mechanism; the success in protection depends entirely upon a balance in quantity. If the proliferation inaugurated by the calcium chloride (or other) activator is inhibited by the neutralization of all the toxin produced as it is secreted, the gangrene will be limited to the area of the necrosis, the course of which will be arrested and the natural healing processes will set in. If the proliferation is so intense that the toxin is not neutralized as formed, the gangrene spreads and the concentration of toxin rises, producing the well-known rapid and fatal result.

In a natural infection the means of proliferation are primarily independent of the presence of toxin, however important this substance becomes once the gangrene is set up. In this early period the action of an antibacterial serum in attacking the proliferating bacteria seems to be of paramount importance. Thus in the experiments described above such a serum administered previous to the infection is capable of so inhibiting the multiplication of the bacteria that they are unable to produce an amount of toxin sufficient to set up a generalized gangrene. Here, just as in the example sketched above with the antitoxin, the infection is limited to the area necrosed by the activator, and the local lesion is in due course healed by the natural capacity of the body. It must be emphasized that there is no neutralizing power whatsoever against the toxin in the "O" immune serum. Its action in protection is rendered impressive when it is remembered that the control animals die within 16 hours with a fulminating gangrene implying a high production of toxin.

We would deprecate any implication that these results discount the great importance of the highest possible antitoxic content in an immune serum. It is obvious that the ideal serum, whether used prophylactically or curatively, should contain both these antibodies in effective amounts. The "O" immune serum should be made with a mixture of all four types unless the hyper-immunization can be driven to such a point that the antigen common to all the types finds effective expression in the immune body content.

The bearing of this work upon the sera used against other organisms which

produce toxin is obvious, and suggests that the limitation of essential antibody content to that of a pure antitoxin might require reconsideration.

SUMMARY.

1. A serum which contained only the "O" immune body to a single strain of *Vibrion septique* ("Pasteur III," Group I) was made by injecting bacilli heated to 100° C. intravenously into a horse. This serum was shown to be devoid of antitoxin.

2. This serum given two days previously protected mice against the injection of washed spores accompanied by calcium chloride as activator.

3. While the life of the animals was preserved a local gangrene nevertheless developed from which the animals recovered completely.

4. Two high-power antitoxic sera were unable to save the life of animals against an infective attack which could be resisted by means of the "O" immune serum. Against a lesser attack however, these sera were effective, reducing, just as in the case of the "O" serum, the rapidly fatal gangrene to a local lesion of varying severity from which the animals recovered.

5. The two antitoxic sera showed a very great difference in the "H" antibody content. This striking discrepancy found no expression in the protective action.

6. The "O" immune serum was found to be type-specific.

7. The bearing of these various points on the action of antitoxic and antibacterial sera is discussed.

APPENDIX I.—*Preparation of the Vaccine for the Immunization of the Horse "Vibrio."*

Cultures of *Vibrion septique* (strain "Pasteur III") of 48-hours' growth in glucose broth were centrifuged, the clear broth was removed and the sediment was twice washed on the centrifuge with fresh sterile distilled water. The sediment was resuspended in distilled water so that the product of the bacillary growth of one litre of broth was contained in 50 c.c. of distilled water. This suspension was heated to 100° C. for one hour, after which a sterile 10% solution of NaCl was added, making the NaCl content up to 0·85%. The steaming was then repeated on two successive days, on each occasion for half an hour.

APPENDIX II.—*Preparation of the Spores used in the Infecting Experiments.*

The bacilli are grown in glucose broth for 2 days. The culture is spun down under sterile conditions in the centrifuge and the sediment washed four times in distilled water; the water is removed so that the spores are only just covered with fluid. In this state they are kept in the cold room until they are required for use—usually a period of a few days. The spores are then suspended in distilled water in approximately opacity No. II of the Burroughs, Wellcome & Co.'s series. They are heated in this dilution for one hour at 78° to 79° C. Thereafter they are spun down, the fluid is removed and the sediment is suspended in saline and matched with opacity II.

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IMMUNITY TO THE SALIVARY VIRUS OF GUINEA-PIGS STUDIED IN THE LIVING ANIMAL, AND IN TISSUE-CULTURE.

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THE existence of the salivary virus of guinea-pigs was brought to light by Cole and Kuttner (1926). In 84% of apparently normal adult guinea-pigs in New York they found swollen cells in the ducts of the submaxillary salivary glands. The nuclei of these cells contained large oxyphilic inclusion bodies, similar to those associated with infection by Virus III of rabbits or herpes virus; those of the guinea-pig virus were, however, much larger, and when stained with eosin took on a darker red colour. These changes were hardly ever present in the salivary glands of guinea-pigs less than a month old, but when such young guinea-pigs were inoculated subcutaneously with an emulsion of infected glands typical lesions appeared in their salivary glands 9 to 15 days later. Seven passages in series were effected through young guinea-pigs by inoculating salivary gland material either direct to salivary gland or subcutaneously; in the latter case the salivary gland of the subcutaneously infected animal was used for the next subcutaneous inoculation and so on (Kuttner, 1927). Emulsions of infected salivary glands when introduced intracerebrally into young guinea-pigs produced acute meningitis; the animals

usually died with fever, convulsions and weakness in about 5 days. Intranuclear inclusions were found in mononuclear cells in the meningeal exudate in such animals, and they could also be found locally after injection of the virus into the tongue, lung or testis. These inclusions differed from those found in the salivary ducts in that they were smaller, were contained in cells of normal size, and were stained pink rather than dark red by the eosin. Curiously enough, it was not found possible to effect regular serial passages from brain to brain nor from testis to testis; passages could only be accomplished when salivary gland material was used for transfer. The immunological findings of Cole and Kuttner will be referred to later. For other information concerning the properties of the virus and for figures of the inclusion bodies the reader is referred to the papers of Cole and Kuttner (1926) and Kuttner (1927).

OCCURRENCE OF THE VIRUS IN ENGLAND.

In a stock of guinea-pigs at the National Institute for Medical Research, Hampstead, London, I found intranuclear inclusions in the gland-ducts in 6 out of 19 adult animals (32%). The virus was also apparently present in another stock of guinea-pigs at the Medical Research Council's Farm Laboratories at Mill Hill, but no quantitative data are available as to its incidence. I have also confirmed and in a few respects extended the findings of Cole and Kuttner as to the behaviour of the virus. One or two observations seem worthy of brief record, though they do not bear directly on the immunological observations which are the main purpose of this paper.

Spinal cord lesions.—The first symptoms noted in many young guinea-pigs inoculated intracerebrally were flaccid paralysis of the hind legs and paralysis of the bladder and rectum. It therefore seemed likely that the lumbar cord was inflamed as well as the brain. Histological study confirmed this idea. A mononuclear meningitis was almost invariably present in the lumbar cord of guinea-pigs inoculated intracerebrally; there was some perivascular cuffing and infiltration around the central canal. The nerve-cells in both anterior and posterior horns showed vacuolization and "satellitism." Intranuclear inclusions were present in the cells of the meningeal exudate, though they were less numerous than in the brain. Similar but less intense lesions were present in the cervical and dorsal regions of the cord. The only guinea-pig in which they were examined showed intense mononuclear infiltration in the spinal ganglia, but no lesions were found in the sciatic or brachial nerves. In one guinea-pig encephalitis was unusually severe, and a few nuclear inclusions were found in nerve-cells in the brain as well as in the meningeal exudate. It is interesting that symptoms referable to the lumbar cord were the first to appear in many animals inoculated directly into the brain.

Cytoplasmic changes in salivary duct-cells.—It was further noted that in infected cells of the ducts of the salivary glands, the cytoplasmic changes were often as remarkable as those of the nucleus. Giemsa's stain showed very clearly within the cytoplasm of these enlarged cells a number of rounded blue bodies of fairly uniform size, each contained within a vacuole; they formed a sharp contrast to the pink intranuclear inclusion. Stains of the cytoplasmic bodies for calcium, iron and hyalin were negative, but since they took on a deep red colour with mucicarmin their composition was probably allied to that of

mucin. They were present in ducts both of mucous and serous glands; in the former situation their colouring with mucicarmin was more intense. In guinea-pigs infected but a short time previously typical changes were present in the nuclei, but none in the cytoplasm; the latter, therefore, probably represent some secondary change.

IMMUNITY TO THE SALIVARY VIRUS.

The virus is remarkable in that, as Cole and Kuttner showed, it persists apparently indefinitely in the salivary glands of a guinea-pig which has once been infected, and inclusions can always be found in the gland of such an animal. These guinea-pigs are, however, immune to the effects of virus introduced intracerebrally or elsewhere in the body. It seemed to me important to discover whether or not these carriers of the virus had any demonstrable antibodies in their blood. Kuttner performed a few experiments in which she incubated the serum of infected animals with virus and then inoculated the mixtures intracerebrally into young guinea-pigs. She could not satisfy herself that any antibody was present, but attributes this to the fact that "quantitative factors have not been adequately controlled" (Kuttner, 1927).

I have tried to demonstrate the presence of antibodies by three methods: (i) by inoculating serum-virus mixtures intracerebrally into young guinea-pigs, as Kuttner did; (ii) by inoculating serum and virus subcutaneously, killing the guinea-pigs after 15 days and examining their salivary glands histologically; (iii) by the use of immune serum in "tissue-cultures" of the virus.

General Technique.

A supply of the virus was always available. It was only necessary to inoculate a number of guinea-pigs subcutaneously, and one could rely on recovering virus from their salivary glands when they were killed even months later. Subcutaneously inoculated animals had some fever but no other symptoms. Only once was the gland of such a guinea-pig found non-infective; it was tested 4½ months after infection. Whenever an infected guinea-pig was killed, blood could be collected and the filtered serum used as "immune serum." The immune sera used in the experiments to be described were from guinea-pigs inoculated 23 to 105 days previously. "Normal" guinea-pig serum was obtained from animals less than 3 weeks old. Tissues studied histologically were fixed in Bouin's solution and stained with hæmatoxylin and eosin; toluidine blue and eosin were, however, used for sections of the central nervous system.

1. *Intracerebral Tests.*

Technique.—In this series of experiments an infected guinea-pig was killed, and its salivary glands removed aseptically, minced, measured with a Bashford tumour syringe, and then ground with Ringer's solution or serum as the case might be. Virus thus obtained was left in contact with immune serum for varying lengths of time, and then 0·2 to 0·25 c.c. of the mixture was inoculated intracerebrally with a gauge 20G needle into guinea-pigs of about 3 weeks old (140–180 gm.). Ether anæsthesia was used, but no trephine was

TABLE I.—*Intracerebral Neutralization Tests.*

Guinea-pig.	Amount of virus.	Virus mixed with—	Details of contact of virus and serum.	Test animal. Max. temp.	Symptoms.	Survival or death.	Comment.
Exp. 1:							
154	0·25 c.c. 1:40	I.S. 131	2½ hrs. at R.T.	103°	0	S	Infected gland ground with serum.
155	,, ,,	,,	,,	104·1°	0	S	
156	,, ,,	Ringer	,,	105·4°	+	† 7 days	Infected gland ground with Ringer.
157	,, ,,	,,	,,	105°	+	† 7 ,,	
Exp. 2:							
158	0·2 c.c. 1:40	Ringer	1 hr. at R.T.	104·7°	+	† 3 days	...
160	,, ,,	I.S. 124	No contact	106°	+	† 3 ,,	Infected gland ground with immune serum.
162	,, 1:400	,,	2 hrs. at R.T.	105·4°	+	† 6 ,,	
163	,, 1:40	,,	,,	105°	+	† 6 ,,	
Exp. 3:							
164	0·25 c.c. 1:400	I.S. 127	10 mins. at R.T.	106°	+	† 8 days	Infected gland ground with I.S. or N.S. Then after incubation (in guinea-pigs 164, 166 and 168) diluted 1:10 with Ringer.
165	,, 1:40	,,	,,	105·8°	+	† 6 ,,	
166	,, 1:400	Normal serum	2½ hrs. at R.T.	106·4°	+	† 8 ,,	
167	,, 1:40	,,	,,	106°	+	† 7 ,,	
168	,, 1:400	I.S. 127	,,	105·5°	+	S	
169	,, 1:40	,,	,,	104·4°	+	S	
Exp. 4:							
170	0·25 c.c. 1:20	Ringer	2½ hrs. at R.T.	105·6°	0	S	170 was probably naturally immune: see text.
171	,, ,,	,,	,, 37°C.	105·6°	+	† 6 days	In this experiment infected gland was ground with Ringer and mixed with serum (or more Ringer) —not ground directly with immune serum.
172	,, ,,	I.S. 115	,, R.T.	106°	+	† 6 ,,	
173	,, ,,	,,	No contact	105·8°	+	† 6 ,,	
174	,, ,,	,,	2½ hrs. at 37°C.	105·1°	+	† 6 ,,	
175	,, ,,	,,	No contact	105·6°	+	† 5 ,,	

Exp. 5:								
176	0·25 c.c.	1:40	Ringer	2 hrs. at R.T.	105·4°	+	† 7 days	Virus ground with Ringer; mixed with more Ringer.
177	„	„	I.S. 143–4–5–6	„	105·9°	+	† 6 „	Virus ground with Ringer; mixed with I.S.
178	„	„	„	No contact	106·3°	+	† 6 „	Ditto.
179	„	„	„	2 hrs. at R.T.	104·4°	0	see comment	Virus ground directly with I.S.
180	„	„	„	No contact	105·7°	+	† 6 days	Ditto 179 had no symptoms, but died suddenly on the 5th day. Histologically, no evidence of meningitis.
Exp. 6:								
185	0·25 c.c.	1:20	I.S. 143–4–5–6	2½ hrs. at R.T.	104·8°	+	† 8 days	Virus ground with I.S.
186	„	„	„	„	104·6°	+	† 9 „	
187	„	„	„	No contact	103·7°	0	S	
188	„	„	„	„	105°	+	†10 days	
189	„	„	Ringer	2½ hrs. at R.T.	104·8°	+	† 6 „	...
190	„	1:400	„	„	104·5°	+	† 8 „	...
191	„	„	I.S. 143–4–5–6	„	105°	0	S	Virus ground with Ringer; then mixed with I.S.
192	„	1:40	„	„	105°	+	† 6 days	
Exp. 7:								
195	0·25 c.c.	1:200	Ringer	4½ hrs. at R.T.	104·7°	+	† 7 days	...
196	„	„	„	„	104·9°	+	† 6 „	...
197	„	„	I.S. 143–4–5–6	„	103·3°	0	S	Virus ground with Ringer; then mixed with I.S.
198	„	„	„	„	104°	+	†11 days	
199	„	„	„	No contact	104·6°	+	†11 „	
200	„	„	„	„	104°	+	† 8 „	

In the column relating to survival or death S = survival. † 6 days = animal died 6 days after inoculation, etc.
I.S. = Immune serum. N.S. = Normal serum.

necessary with animals of this size. The temperatures and symptoms of the animals were recorded daily.

It had been noted that fully formed inclusions could not be found in the salivary gland in less than 10 days after subcutaneous inoculation; the same was apparently true of guinea-pigs inoculated intracerebrally in a dose insufficient to kill. Occasionally a round pink body was present in a vacuole within the nucleus, but by no means occupying the whole nucleus; I have considered such an appearance as an "early form" of an inclusion body. When an animal survived an intracerebral test without showing symptoms it was killed about 10 days after inoculation and its salivary gland examined histologically. If fully-formed inclusions were numerous it was concluded that its failure to take was due to pre-existing infection (in fact this was only found with one animal—No. 170, Exp. 4). If there were no inclusions, or at most a very few "early forms," it was concluded that there was no pre-existing infection or natural immunity and that the animal's survival was due to the immune serum used.

Results.—It was found that some neutralizing power of the serum was demonstrable in most experiments; it could be shown most readily when infected gland was ground up in the mortar directly with the immune serum and allowed to stand for some time, no diluent at all being used. A clean result of this kind is seen in Exp. 1, Table I. In Exp. 2 the virus used was very potent, killing a control in 3 days; at most it can be said that those animals receiving the immune serum survived twice as long as that. In Exp. 3 the serum apparently saved the guinea-pigs when it was in contact with the virus for 2½ hours at room temperature before injection; 10 minutes' contact did not suffice. The rapid death of guinea-pig 160 (Exp. 2) also suggests that some contact of serum and virus before injection is desirable. The neutral mixture which did not kill guinea-pig 169 was diluted ten-fold with Ringer's solution, in order to see whether the virus could be reactivated by dilution as is possible with serum-virus mixtures in experiments with fowl-plague (Todd, 1928), vaccinia (Andrewes, 1928) and herpes (Bedson, 1928). The survival of guinea-pig 168 shows that no such reactivation occurred in this experiment with the guinea-pig virus. Normal guinea-pig serum was used instead of Ringer's solution for the control animals in Exp. 3. In Exp. 4 the infected gland was ground up with Ringer's solution and then mixed with immune serum, instead of grinding the gland directly in the immune serum: no neutralization could be demonstrated. The survival of one control (170) may reasonably be ascribed to a pre-existing infection, as mentioned earlier. Exp. 5 was unsatisfactory, but may probably be taken as confirming the desirability of grinding the virus in undiluted serum and then letting the mixture stand. No. 179, which received virus so treated, remained perfectly well when all the other guinea-pigs had obvious encephalitis; then it abruptly died of some other cause. Exps. 6 and 7 show, however, that provided a dilute virus is used it is possible to obtain neutralization, even though the infected gland is not ground directly with the serum. It is also noteworthy that, contrary to the general rule, guinea-pig 187 was saved, even though a freshly made serum-virus mixture was injected.

Comment.—These experiments show many irregularities, but nevertheless

do suggest that the sera of infected guinea-pigs possess neutralizing properties. After all, experience with herpes and vaccinia (Andrewes, 1928) shows that immune bodies against a virus, even when plentiful, are hard to demonstrate if intracerebral inoculations are employed.

2. *Subcutaneous Tests.*

Attempts were next made to prevent the virus from localizing in the salivary gland when injected subcutaneously, either by mixing it with immune serum before inoculation, or by giving immune serum to the guinea-pig at another site. The results of four such experiments are recorded in Table II: in all cases the guinea-pigs were killed after about 15 days and their salivary glands examined histologically for inclusions.

TABLE II.—*Subcutaneous Tests.*

Guinea-pig.	Dose of virus.	Serum used.	Animal killed after—	Inclusions in salivary gland.	Comment.
Exp. 8:					
209	0·5 c.c. V. 1:1000	3 c.c. 203–208	18 days	+	Serum given subcutaneously 23 hours before virus.
210	,, ,,	,, ,,	,,	+	
211	,, ,,	,, ,,	,,	++	
212	,, ,,	0	,,	++	Controls. No serum.
213	,, ,,	0	,,	++	
214	,, ,,	0	,,	++	
215	,, ,,	3 c.c. 203–208	,,	±	Serum mixed with virus. Contact for 5 hours at room temperature.
216	,, ,,	,, ,,	,,	0	
217	,, ,,	,, ,,	,,	0	
218	,, ,,	,, ,,	,,	++	Serum inoculated into 1 flank; virus immediately after into the opposite flank.
219	,, ,,	,, ,,	,,	+	
220	,, ,,	,, ,,	,,	+	
Exp. 9:					
246	0·25 c.c. V. 1:500	2·75 c.c. pooled serum	15 days	0	Serum mixed with virus. Contact for 5 hours at room temperature.
247	,, ,,	Ditto	,,	0	
248	,, ,,	,,	,,	0	
249	,, ,,	,,	,,	0	Serum mixed with virus and inoculated forthwith.
250	,, ,,	,,	,,	0	
251	,, ,,	,,	,,	0	
252	,, ,,	0	,,	+	No serum control.
Exp. 10:					
260	0·5 c.c. V. 1:200	0·5 c.c. 239–244	15 days	± (very early forms)	Serum and virus in contact 4 hours at 37° C.
261	,, ,,	,, ,,	,,	± Ditto	
262	,, ,,	,, ,,	,,	± ,,	
263	,, ,,	,, ,,	,,	± (early forms)	Virus kept 4 hours at 37° C., then mixed with immune serum and inoculated forthwith.
264	,, ,,	,, ,,	,,	± ,,	
265	,, ,,	,, ,,	,,	+	
266	,, ,,	0	,,	+	No serum controls. Virus kept 4 hours at 37° C., then diluted 1 in 2 with Ringer.
267	,, ,,	0	,,	++	
268	,, ,,	0	,,	++	

In the column relating to inclusions 0 = none found. ± = very few early forms.
+ = moderate numbers. + + = inclusions abundant.

Results.—Exp. 8 (Table II) makes it clear that when serum and virus are inoculated into opposite flanks no protection at all results—not even (*v.* guinea-pigs 209, 210, 211) when the serum is given 23 hours ahead of the virus. This plan was followed in another experiment; as the results were quite negative, the protocol has not been included in Table II. The serum thus differs from sera active against foot-and-mouth disease or vaccinia in that it does not in small doses prevent "generalization" to a part of the body remote from the site of inoculation. But when serum-virus mixtures were injected, some neutralization apparently did occur (Exps. 8, 9 and 10). Exps. 9 and 10 were designed to show whether or no it was necessary to keep the serum and virus in contact outside the body before inoculation. Apparently this was not essential, though in Exp. 10 there was perhaps a little better neutralization when serum and virus were kept in contact. Evidently, then, such action as the serum has is exerted either *in vitro* or at the site of inoculation by preventing the virus from obtaining a local foothold; there is no reason to believe that serum in the doses used can confer a passive protection on the salivary gland after diffusion throughout the body.

3. *Tissue Culture.*

Technique.—An attempt was made to cultivate the virus in a slight modification of Maitland's medium (Maitland and Maitland, 1928), such as had proved efficacious in the cultivation of Virus III of rabbits (Andrewes, 1929[2]). Small pieces of minced guinea-pig tissue were placed with a platinum spatula in the bottom of a 5 cm. Carrel flask. To this was added 0·5 c.c. of serum, 1·5 c.c. of Tyrode's solution and 0·1 c.c. of virus-emulsion. Where immune serum was used it was always added before the virus. The necks of the flasks were covered with rubber caps and the flasks incubated at 37° C., usually for 4 days. It was found that guinea-pig salivary gland (the source of virus) was not so readily obtained in a sterile condition as rabbit's testis, which had been used for the cultures of Virus III. Moreover, even when it was sterile, guinea-pig tissue did not regularly survive as well in the serum-Tyrode medium as did rabbit testis; only the testes of very young guinea-pigs could be relied upon to do so. At first the normal serum used was obtained from 3-week-old guinea-pigs. Since this was hard to obtain in any quantity, normal rabbit serum was used later; the guinea-pig cells survived in this as well as in guinea-pig serum. In any case, this method merely permits the survival of cells at the periphery of a tissue fragment; a few mitoses may be found, but there is no true tissue culture.

Development of inclusion-bodies in tissue-cultures.—In many cultures intranuclear inclusions staining pink with eosin appeared in mononuclear cells in the interstitial tissues. As Table III shows, they could always be found in cultures of testis in normal serum, but were less constant in cultures of ovary, brain or salivary gland, perhaps because the cells of these did not survive so well. They resembled the inclusions described by Cole and Kuttner in the brain, lung, tongue and testis; they were never so large nor so darkly staining as those in the salivary duct-cells. They were usually numerous in cultures incubated for 3, 4, 5 or 6 days, but were doubtfully present in cultures only

48 hours old. Though their final appearance was much like that of virus III or herpes inclusions, their development seemed rather different. In the early stages of formation of virus III or herpes inclusions, the nucleolus becomes unrecognizable, the whole nucleus takes on a homogeneous purplish stain with hæmatoxylin and eosin, and the nuclear membrane becomes thickened and beaded (see figures in Lauda and Luger, 1926). In cells affected by the guinea-pig salivary virus the first change seems to be the appearance of a small, eosinophilic body within a vacuole in the nucleus well away from the unchanged nucleolus. This pink-staining body gradually enlarges and pushes the nucleolus aside.

Thirty-one cultures of normal guinea-pig tissue (19 of testis, 4 of ovary, 8 of salivary gland) had no virus added to them; none of them showed any inclusions on histological examination. Nor did inclusions appear in a culture of normal rabbit testis inoculated with virus; the rabbit is not susceptible to this virus.

Attempted subculture of the virus.—In primary cultures the virus survived for at least 5 days; it was recovered by inoculating young guinea-pigs subcutaneously and examining their salivary glands 15 days later. In two flasks containing no tissue it did not survive 5 days, but it was recovered from one culture in which the tissue had necrosed and no inclusions had formed and from another in rabbit testis from which also inclusions were absent. It is therefore not certain how the presence of tissue enabled the virus to remain alive. Although nuclear changes were regularly produced—at any rate in cultures of testis—there was no evidence that the virus multiplied. All attempts to subcultivate the virus were negative, whether from cultures of salivary gland to salivary gland, salivary gland to testis, testis to salivary gland or testis to testis. No inclusions were found in any one of 12 subcultures examined. Four such subcultures were inoculated into young guinea-pigs, but proved to contain no living virus. This failure is at first surprising, but is, perhaps, less so if it is remembered that Cole and Kuttner failed to propagate the virus from testis to testis in the living animal. It is possible that the attempt at subculture might have been successful if it had been found possible to keep cultures of salivary gland alive long enough to enable the large inclusions to form in the duct-cells.

Cultures in tissues of immune animals.—In earlier experiments with virus III, I found (Andrewes, 1929[3]) that this virus would multiply and form inclusions in cultures of testis from an immune rabbit, provided that the serum of a normal rabbit was used. The guinea-pig virus also proved capable of forming inclusions in cultures of the tissues of an infected animal. While virus III formed inclusions in cultures of immune testis, it did not do so every time, and the inclusions were always scarcer than when normal testis was used. The salivary virus, on the other hand, formed inclusions as readily and as regularly in immune tissues as in those of an uninfected guinea-pig 3 weeks old (see Table III). The testis of an infected guinea-pig may fairly be counted as immune since virus inoculated into it *in vivo* produced no inclusions—nothing, in fact, beyond a little mononuclear reaction, which was possibly allergic in nature.

Cultures in immune serum.—It will be seen from Table III that when

grown in normal serum the virus formed inclusions in 100% of cultures of testis and in at least 50% of those of other tissues. But when the serum of an infected guinea-pig was used inclusions never developed. Fuller protocols are given in Table IV of 3 experiments with testis; these are the most important, since with this tissue inclusions always formed in the control cultures in normal serum.

TABLE III.—*Summary of Results of Tissue-Cultures.*

Tissue.	Serum.	Number of cultures examined for inclusions.	Number positive.	Number negative.
Normal testis	Normal (rabbit or young guinea-pig)	14	14	0
Immune „	Ditto	4	4	0
Normal salivary gland	„	9	6	3
Immune salivary gland	„	6	3	3
Normal ovary	„	2	1	1
Immune „	„	2	1	1
Normal brain	„	2	1	1
Normal testis	Immune guinea-pig	3	0	3
Immune „	„ „	1	0	1
Normal salivary gland	„ „	1	0	1
Immune salivary gland	„ „	4	0	4
Immune ovary	„ „	1	0	1

TABLE IV.—*Effect of Immune Serum on Inclusion Formation.*

Culture.	Testis.	Serum.	Inclusions.
S5 (3)	Normal	Normal rabbit	+
„ (4)	„	„ „	+
„ (5)	„	Immune guinea-pig	0
S12 (5)	Normal	Normal rabbit	+
„ (6)	„	„ „	+
„ (7)	„	Immune guinea-pig	0
S13 (6)	Normal	Normal rabbit	+
„ (7)	„	„ „	+
„ (8)	„	Immune guinea-pig	0

DISCUSSION.

The intracerebral tests described gave irregular results, but taken together, do suggest that a guinea-pig carrying the virus in its salivary glands has neutralizing properties in its serum. The subcutaneous technique shows the same thing rather more clearly. Tissue culture methods demonstrate quite

cleanly that neutralizing properties are present in such sera. This is of interest in relation to the question of the persistence of viruses in immune animals, and lends support to the idea that a virus which is intracellularly situated may be protected from the action of an immune serum (Andrewes, 1929[1]; Rivers, Haagen and Muckenfuss, 1929). In tissue cultures the immune serum seems to prevent the virus from forming inclusions in cells. *In vivo* all the guinea-pigs' tissues are quickly immunized except the cells of the ducts of the salivary glands. One can only speculate wildly as to how and why the virus manages to maintain itself there and there only.

The formation of inclusions *in vitro* in immune as well as in normal tissues has been discussed in an earlier paper (Andrewes, 1929[2]). Unpublished experiments of mine with the virus of herpes show that the same thing occurs with this virus; and Rivers, Haagen and Muckenfuss (1929) found that Guarnieri bodies would form *in vitro* in cultures of immune rabbit cornea.

The inclusions produced by the salivary virus in testis and brain seem to be of different significance from those due to Virus III. With the latter virus the infectivity of a tissue usually goes parallel with the number of inclusions it contains, and the inclusions give an indication that the virus is multiplying. This may also be true as regards the large inclusions in the salivary ducts of guinea-pigs, but the inclusions in the interstitial tissues of the testis or in the meninges seem to denote merely a reaction to the presence of the virus and may have nothing to do with its multiplication.

SUMMARY AND CONCLUSIONS.

1. Guinea-pigs harbouring the salivary virus in their salivary glands have neutralizing properties in their serum. This statement is based on the following evidence:

(*a*) Virus mixed with immune serum and inoculated intracerebrally into young guinea-pigs is, under appropriate conditions, partly or wholly neutralized and fails to kill.

(*b*) Virus mixed with immune serum and inoculated subcutaneously into young guinea-pigs is partly or wholly neutralized, so that inclusion bodies are found scantily or not at all when the salivary glands are examined subsequently.

The evidence cited under (*a*) and (*b*) is in itself far from conclusive, but is probably significant in view of the much sharper results obtained with the method of tissue culture.

(*c*) In the presence of immune serum the virus does not produce inclusion bodies in "cultures" of guinea-pig testis or other tissue in Maitland's medium.

2. When normal serum is used the salivary virus will regularly cause the appearance of inclusion bodies in the interstitial cells of testis grown in Maitland's medium; less regularly it causes them to appear in cultures of salivary gland, ovary or brain. In spite of this, it does not appear that the virus multiplies in this medium.

3. The inclusions form just as readily in cultures of immune tissues as of normal tissues, provided that normal serum is used.

I wish to thank my colleague, Dr. J. R. Perdrau, for advice and assistance with the histological observations.

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THE IMPORTANCE OF SOMATIC ANTIGEN IN THE PRODUCTION OF AERTRYCKE AND GÄRTNER IMMUNITY IN MICE.

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AERTRYCKE IMMUNITY.

IN a previous paper the writer, in collaboration with H. M. Ibrahim (1928), compared the immunogenic values of the H and O antigens of a *Salmonella aertrycke* vaccine in mice, and concluded that while H antigen, obtained by employing a rough motile strain, was ineffective as an immunizing agent, O antigen, obtained by heating a smooth, motile strain to 100° C. for one or two hours, was not as potent as the antigen contained in the same smooth, motile strain heated only to 60° C., and suggested that a probable cause was the damage wrought upon the O component by the prolonged heating to 100° C. and not the lack of the H component in the vaccine prepared in this way.

To verify this probability advantage was taken of the acquisition of a natural O form of the Aertrycke strain.

The isolation of this O variant occurred in the following way: Over three months after the giving of the test dose in the immunity experiment alluded to above there still remained 15 survivors, and it was decided to investigate what proportion of these were carriers. The animals were gassed, and specimens of spleen, liver, kidney and heart blood were taken from each

and incubated in broth at 37° C. Of these 15 survivors, it was in this way found that 6 were carriers of *S. aertrycke*, the bacillus being isolated in pure culture from the spleen in all 6 mice, from the liver in 4, from the heart blood in 2 and from the kidney in 1 animal. From one of the rats which yielded *S. aertrycke* in both spleen and liver cultures the O variant was obtained, the liver culture being a pure culture of that form while the spleen culture represented the normal motile H + O variety. Proof that the former strain was indeed an O form was obtained not only by its lack of motility but also by serological methods; this variant neither agglutinated with, nor absorbed, nor, when injected into a rabbit, occasioned the formation of H agglutinins.

It was with these two smooth Aertrycke strains that the experiment to be described was carried out.

Preparation of Vaccines.

Vaccines were prepared by growing cultures on agar at 37° C. for 24 hours, washing down in saline, counting, sterilizing (on the average in a density of 15,000 million organisms per c.c.), and diluting with saline to inoculation strength. Sterilization was effected not only at a temperature (60° C. for 1½ hours) which does not destroy the agglutinogenic properties of H antigen, but also, in the case of the O vaccine, at 100° C. for short and long periods to test the theory that a temperature of this elevation is detrimental to the immunizing powers of somatic antigen; in the case of the motile variant, sterilization by chloroform (10% added to the saline suspension in a corked vessel, followed by incubation at 37° C. for 24 hours) was also practised, as by this method the flagellar antigen appears to be less altered than it is by heating to 60° C.; a suspension heated to 60° C. for 1½ hours agglutinates badly with H antibodies, while treatment with chloroform, as above, leaves the agglutinating properties of the suspension unaltered.

S. aertrycke shares more or less completely its somatic antigen with other Salmonellas; a series of these was chosen, and from them vaccines were prepared as above and sterilized at 60° C.; care was taken that here, as with the Aertrycke strains, only smooth variants were employed, the criteria being not only normal colonial growth and salt stability, but lack of agglutination with R antibodies; these type-alien strains were all of them motile forms.

It may be noted here that while final sterilization carried out at the lower temperature was effected at 60° C. for 1½ hours, all these vaccines had been subjected to a preliminary and ineffectual heating to 56° C. for 70 minutes; this lower temperature had been first chosen to bring the vaccines into line with a stock paratyphoid B vaccine, also included in the experiment, in which ultimate sterility was no doubt achieved by the addition of 0·5% phenol consequent to heating.

In this way some eleven vaccines were obtained; they are enumerated in Table II, the somatic antigen factors each strain contains according to White (1926) being indicated alongside, together with the reference number the strain bears in the catalogue of the National Collection of Type Cultures.

The extent to which the various somatic factors are shared by the bacteria used is indicated by the cross-agglutination obtained with an O serum prepared from the Glasgow O variant. This serum had a titre of 1/6400 for

itself; calling this 100, one gets the cross-agglutination values shown in Table I.

TABLE I.

S. aertrycke "Glasgow" (O variant) . . .	= 100
" " (H + O variant) . .	= 100
S. paratyphi B "Tidy"	= 100
S. reading "Reading"	= 12
S. newport "Newport"	= 12
S. typhi "Mrs. S—"	= 6
S. suipestifer (European variety) "New Haw" .	= 0

While paratyphoid B agglutinates to titre limit, Reading, Newport and typhoid strains are only slightly influenced; there is a definite but inconsiderable overlapping of somatic antigen factors; the suipestifer strain shows no community of antigen at all.

Immunization of Animals.

Mice were employed, 20 being immunized with each vaccine. They were kept in pairs in glass jars, and fed on bran, oats, bread and occasional greenstuff with a supply of water.

Three subcutaneous inoculations were given in the following manner: 200 million organisms, 7 days' interval, 800 million, 14 days' interval, 3000 million. After a further interval of 18 days the test-dose was administered; it consisted of $\frac{1}{2}$ c.c. of a 1/20 broth dilution of a 24-hour broth culture of *S. aertrycke* "Glasgow" (a smooth motile variant) injected intraperitoneally, and was given to 20 uninoculated control mice as well; subdivisions of this amount of broth culture were injected into a small series of mice to obtain some idea of the number of m.l.d. which the test-dose contained.

The immunizing doses were tolerated well, only 10 out of the 221 mice dying during the 39 days which elapsed between the commencement of inoculation and the giving of the test-dose. The deaths occurred irregularly among the different vaccine batches. This mortality-rate was not greater than the one which obtained among the mice before immunization was begun; 220 mice had been kept in the bottles for a 21 days preliminary observation, and 8 of them had died; at the end of this time, before immunization, fresh mice were added to replace those that had died. In all cases of death heart blood-cultures were made, the organisms isolated during immunization being generally *B. proteus*, sometimes *B. morgan I* or coliform bacilli; after the test dose *S. aertrycke* was recovered in every case, generally in pure culture, occasionally in association with one of the organisms just mentioned.

The results of the experiment shown in Table III are well defined:

(*a*) Inoculation of mice with three spaced doses of killed vaccine can protect the majority of the animals against a multiple lethal test-dose of living *S. aertrycke* over a period of 32 days so long as there is identity of somatic antigen between the vaccine and the infecting organism and so long as that antigen has not been damaged by prolonged heating to 100° C. (see vaccines 1 to 7).

TABLE II.—*Vaccines used in the Aertrycke Immunity Experiment.*

Vaccine.	Strain.	Variant.	Reference number.	Somatic antigen factors.	Sterilization.
1	Aertrycke "Glasgow"	O	3047	I, II, 7, 8	1½ hours, 60°.
2	„ „	„	3047	„	¼ hour, 100°.
3	„ „	„	3047	„	2 hours, 100°.
4	„ „	H + O	3048	„	1½ „ 60°.
5	„ „	„	3048	„	24 „ 37°, chloroform.
6	Paratyphoid B "Tidy"	„	14	„	1½ „ 60°.
7*	„ „	„	14	„	70 minutes, 56°.
8	Reading "Reading"	„	72	II, 7, 8	1½ hours, 60°.
9	Newport "Newport"	„	129	IV, VI, 7	„
10	Typhoid "Mrs. S—"	„	2843	III, 8	„
11	Suipestifer "New Haw"	„	1773	V, VI	„

* This stock vaccine had stood, since its manufacture, in the cold room of the Lister Institute for 9 months in the presence of 0·5% phenol.

TABLE III.—*Mice Immunized Subcutaneously with 200 × 10^6, 800 × 10^6 and 3000 × 10^6 Organisms, with Intervals of 7 and 14 Days.*

Vaccines.	Number of mice tested.	Total number of mice dead, in days after test-dose.										Average days till death.	Percentage of survivors after 32 days.
		1.	2.	3.	4.	5.	6.	7.	8.	9.	20.	32.	
1	20	0	0	0	0	2	2	2	3	3	5	6	13·0	70
2	18	0	0	1	1	1	1	1	1	1	4	5	15·0	72
3	20	4	7	7	7	7	9	9	9	9	14	15	6·7	25
4	19	0	0	0	1	1	2	2	2	2	3	6	15·8	68
5	19	0	0	0	0	1	1	2	2	3	6	7	13·1	63
6	17	0	2	2	2	2	2	3	3	3	7	7	9·3	59
7	18	1	2	2	2	2	2	3	5	5	7	8	11·4	56
8	20	7	12	13	14	17	17	17	18	18	18	19	4·2	5
9	20	8	17	18	18	18	18	18	19	19	19	19	2·0	5
10	19	3	7	8	11	12	14	16	16	17	18	18	4·6	5
11	20	0	11	12	15	17	17	17	17	17	18	18	3·2	10
Controls:														
Full test-dose	20	7	14	16	18	18	18	18	18	19	19	19	2·3	5
1/5 „	2	0	0	2	2	2	2	2	2	2	2	2	...	...
1/50 „	2	0	0	0	0	0	0	0	0	0	2	2	...	...
1/500 „	2	0	0	0	1	1	1	1	1	1	2	2	...	...
1/5000 „	2	0	0	0	0	0	0	1	1	1	1	1	...	...

Test-dose = ½ c.c. of 1/20 dilution of 24 hours' broth culture of *S. aertrycke* (smooth motile variant), injected intraperitoneally 18 days after last immunizing dose.

(*b*) There is no difference in response to a somatic vaccine heated to 60° for 1½ hours and one heated to 100° for ¼ hour, but when the exposure to 100° lasts as long as 2 hours, the immunogenic value of the vaccine suffers considerably and the percentage of animals protected falls from 70 to 25 (see vaccines 1, 2 and 3).

(*c*) With regard to the value of flagellar antigen as a constituent of a prophylactic vaccine, compare vaccines 1, 2, 4 and 5, all prepared from the same strain of *S. aertrycke*. The first two contain no flagellar antigen; the last two possess it. There is no significant difference between 70 and 72% for non-flagellar vaccine and 68 and 63% for vaccine containing flagellar substance. Flagellar antigen does not apparently play a part in the evocation of immunity.

(*d*) That the somatic antigen concerned in the vaccine need not be derived

from the same Salmonella type as that composing the infective dose is demonstrated by a comparison of vaccines 4, 5, 6 and 7. The two former were made from an Aertrycke and gave 68 and 63% protection, the two latter from a paratyphoid B strain and gave 59 and 56% protection. Both these types possess identical somatic antigen as revealed by agglutination and both sets of vaccine give roughly equal results; the immunogenic value of their respective somatic antigens is approximately the same. This is what Topley (1929) found in his extensive experiments on mice, planned on similar lines to these, the outstanding difference being the use of contact infection in the place of intraperitoneal injection as test-dose.

(*e*) Vaccine 7 was introduced into the experiment to test the keeping qualities of Salmonella antigen; it was a stock vaccine which had been kept for 9 months in the cold room in the presence of 0·5% phenol; neither of these factors had sufficed to damage its antigenic value. Vaccine 7 protects 56% of the mice, which is on a par with the 59% of vaccine 6, freshly prepared from the same strain of paratyphoid B.

The keeping qualities of vaccine (in this case typhoid and cholera) over still longer periods has recently been tested by Konrich (1929). He found that storing for 2 or even 3 years in the cold room did not damage their immunogenic properties, 2 years at room temperature only slightly, but after that length of time at 37° C. the vaccines were useless for protective purposes.

(*f*) How far the less related Salmonella types are capable of protecting is indicated by the use of vaccines 8 to 11, which have been arranged in order of their diminishing somatic relationship with *S. aertrycke*, *S. reading* (vaccine 8) agglutinating, it will be recalled, to 12% of the titre of an Aertrycke O serum and *S. suipestifer* (vaccine 11) not agglutinating with it at all; none of these vaccines confers any appreciable immunity upon the inoculated mice, whether judged by the rate of death or the final mortality, as compared with the group of 20 uninoculated controls (see Table III); for qualitative or quantitative reasons, or both, the somatic factors which these strains share with the infecting organism are insufficient to establish any immunity in the inoculated animals. The observations of Perry and Tidy (1919) tend to confirm this; they reported an outbreak in the army of 851 cases of diarrhœa due to *S. newport*, 26% of whom had not been inoculated with T.A.B. vaccine for over 12 months, and came to the conclusion that more recent inoculation with that vaccine had not influenced either the incidence or the severity of the disease.

It will be noticed that in these groups of mice, as in the batch of controls, 1 out of 20 has escaped death; it seems likely that a small percentage of the animals as delivered to the laboratory is normally possessed of this degree of immunity. With regard to the size of the test-dose, it may be said that roughly 500 m.l.d. were contained in it, though the final deaths in the dilution series occurred as late as 11 and 16 days.

Agglutinins and Immunity.

An attempt was made to correlate the presence of somatic antibodies in the sera of the inoculated mice with their subsequent resistance to infection.

During the second week after the last immunizing dose of vaccine the mice were bled from the tail, and by means of a hæmocytometer pipette

measured quantities of blood were added to citrated saline; after centrifugation the supernatants were diluted with saline and tested against suspensions of Aertrycke, both O and H + O variants being used. The sodium citrate contained in the various dilutions did not interfere with agglutination. It was at once seen that the formation of agglutinins varied greatly from mouse to mouse within the same group. To take the case of the O antibodies first, batch 1 had been inoculated with Aertrycke "Glasgow" O, heated for 1½ hours at 60° C., and of the 20 mice tested, 8 failed to agglutinate Aertrycke somatic antigen in a dilution of 1/100, 6 gave a titre of 1/100, 4 went as far as 1/200 and 2 reached a titre of 1/400, which none surpassed; with regard to the formation of H antibodies, the batch inoculated with vaccine 4 which was prepared from Aertrycke "Glasgow" H + O, heated for 1½ hours at 60° C. showed similar variations; of the 18 mice tested, 2 agglutinated in a flocculent manner to 1/400, 2 had a titre of 1/800, 5 reached 1/1600, 7 went as far as 1/3200, and 2 even agglutinated in as high a dilution as 1/12,800. Several of the mice inoculated with O vaccine and giving 1/400 O titres were examined for H agglutinins by absorption of their sera with Aertrycke O and agglutination with Aertrycke H + O strains, but no residual H agglutinins could be detected. As in the preliminary experiments with rabbits, so here in the case of mice, the non-motile Aertrycke variant is revealed as a pure O strain.

A summary of these agglutination results is given in Table IV, the average titre of each group being indicated by taking a positive agglutination at 1/100 as the unit and regarding 1/200 as representing 2 units and so on.

TABLE IV.

Vaccine.	Number tested.	Number of sera giving O agglutination no higher than—				Average agglutination units.		Percentage protected.
		<1/100.	1/100.	1/200.	1/400.	O.	H.	
1. Aertrycke O 1½ hours, 60°	20	8	6	4	2	1·1	...	70
2. „ „ ¼ hour, 100°	18	12	2	3	1	0·7	...	72
3. „ „ 2 hours, 100°	20	19	0	1	0	0·1	...	25
4. „ H + O 1½ „ 60°	19	13	6	0	0	0·3	33	68
5. „ „ 24 „ 37° chloroform	19	3	10	4	2	1·4	30	63
6. Paratyphoid B 1½ hours, 60°	17	16	1	0	0	0·1	...	59
7. „ 70 minutes, 56°	18	14	4	0	0	0·2	...	56
8. Reading 1½ hours, 60°	10	10	0	0	0	0	...	5
9. Newport 1½ „ 60°	10	10	0	0	0	0	...	5
10. Typhoid 1½ „ 60°	10	10	0	0	0	0	...	5
11. Suipestifer 1½ „ 60°	10	10	0	0	0	0	...	10

Very little correlation is visible in Table IV between the average somatic agglutination titre of a group and its resistance to infection, except in the case of the last four alien type-groups; here where there is so little somatic antigen common to the vaccine strain and *S. aertrycke*, no demonstrable Aertrycke O agglutinins have been evoked and no protection has ensued.

Confining one's attention to the vaccines with a common somatic content, one sees vaccine 5 associated with a relatively high titre, but not protecting better than vaccine 4 which occasioned a low titre; a somewhat similar discrepancy is seen between the results with vaccines 1 and 2; vaccines 6 and

7 occasioned an agglutinin response as poor as did vaccine 3, but they afforded much better protection to the mice. In general it can be said that a low somatic agglutinin titre is not necessarily associated with a low degree of immunity. A similiar lack of correspondence was reported by Topley (1929), working with paratyphoid B and Aertrycke inoculations in mice.

The question may be viewed from a different angle by seeing what proportion of mice of the various titres died irrespective of the group to which they belonged. The results are given in Table V, from which the animals belonging to Group 3 have been excluded because in the case of this vaccine a low titre seemed to be definitely associated with poor immunity, both agglutinogenic and immunogenic properties of the vaccine being damaged by heating to 100° for 2 hours.

TABLE V.

Days after test-doses.	Deaths in Batches 1, 2, 4, 5, 6, 7, in mice with somatic titres of—			
	Less than 1/100.	1/100 and higher.	1/200 and higher.	1/400.
4	6 out of 66 = 9%	0 out of 45 = 0 %	0 out of 16	0 out of 5
14	17 „ 66 = 26%	7 „ 45 = 15½%	0 „ 16	0 „ 5
32	25 „ 66 = 38%	14 „ 45 = 31 %	4 „ 16	2 „ 5

Table V brings evidence that there is perhaps a slightly raised degree of resistance to infection in mice with higher titres, and what is more definite, a lag in the rate in which they die; this point is more clearly made by a comparison of the average length of life of the animals which die in the higher and lower titred groups; 14 mice with titres of 1/100–1/400 live on the average 15·1 days; 25 mice with titres less than 1/100 live on the average 11·4 days. This difference in length of life would seem to represent the effect of circulating antibodies upon infection; if the effect is not great, it must be remembered that the agglutinin titres are not of a high order either.

In summary, one may say that there is a basal immunity independent of circulating antibodies. It is related possibly to small undemonstrable amounts of these in the serum, or to antibodies fixed in the tissues, or to a sensitized condition of the antibody-producing cells, which in this state liberate defensive antibodies more rapidly than do normal unsensitized cells, or finally it may not be dependent on antibody formation in any way—some purely cellular immunity. Over and above this basal immunity, it would seem that there is an element of resistance dependent on circulating antibody, to which the O agglutinin titre may serve as a rough index.

As has been constantly noticed, mouse survivors from an Aertrycke test-dose rarely effect a complete sterilization of themselves in respect of that organism and the same observation was made in this experiment. At the end of the 32 days all the mice remaining alive were gassed, and large portions of the spleens, varying from a half to a quarter of the organ, were transferred to broth and incubated at 37° C. for 7 days. It was seen how incomplete an immunity had resulted from the series of inoculations: of the 84 survivors who had had the full test-dose, no less than 71 were carriers of *S. aertrycke*, that bacterium being isolated in pure culture from the spleen. No correlation between sterility of spleen and height of titre before testing for immunity was observed.

GÄRTNER IMMUNITY.

Another experiment planned to establish the importance of somatic antigen for prophylactic purposes was performed on mice, the infecting bacterium this time being *S. enteritidis*, Gärtner; the vaccines were made with (*a*) 2 motile strains and 1 non-motile strain of this organism, (*b*) a motile *S. typhi* which has somatic antigen but no flagellar antigen identical with that of *S. enteritidis*, Gärtner, and (*c*) two antigen alien Salmonellas—*S. paratyphi A* and *S. suipestifer* (European variety), as well as a quite unrelated *Staphylococcus aureus*. The vaccines were as usual 24-hour agar cultures, suspended in saline and sterilized, this time all of them, at 60° C. for 1½ hours. The dosage was 200 million, 10 days' interval, 800 million, 15 days' interval, 3000 million given subcutaneously.

The test-dose consisted of ½ c.c. of a 1/20 broth dilution of a 24-hour broth culture of a motile Gärtner strain heterologous to all used for vaccine purposes, and was administered intraperitoneally 35 days after the last vaccine inoculation. Control mice were injected with this amount at the same time as the inoculated ones, and a rough titration of the test-dose indicated that it represented at least 500 m.l.d.

It was intended that there should be 20 mice in each group, but owing to the numerous deaths that occurred during the period of immunization among the mice set aside as controls, as well as among the inoculated, all groups suffered depletion in numbers. A particularly unsatisfactory feature of the epizootic was the inclusion of *S. enteritidis*, Gärtner, among the organisms isolated from the heart-blood of mice dying before the test-dose was given; on no fewer than eight occasions it was found alone or in association with *B. morgan I*, *B. proteus*, or coliform bacilli which were the organisms generally isolalated.

The test-dose inoculum proved to be a particularly virulent one, 95% of the control mice dying within 2 days and even the immunized mice showing little resistance. Still, the effect of prophylactic inoculation is clearly shown by the increased length of life these mice exhibit.

TABLE VI.—*Mice Immunized Subcutaneously with 200 × 10^6, 800 × 10^6 and 3000 × 10^6 Organisms with 10 and 15 Days' Interval. Test-Dose = ½ c.c. of 1/20 Dilution of 24-hours' Broth Culture of* S. enteritidis, *Gärtner (Smooth Motile Variant), Injected Intraperitoneally 35 Days after Last Immunizing Dose.*

Vaccine.	Variant.	Reference number of strain.	Somatic antigen factors.*	Number of mice tested.	Percentage surviving after—		Average length of life.†
					5 days.	15 days.	
1. Gärtner	H + O	2212	III, 8	18	44	17	7·9
2. „	„	2203	„	15	53	7	6·5
3. „	O	3045	„	14	50	7	7·5
4. Typhoid	H + O	2843	„	13	69	15	7·9
5. Paratyphoid A	„	2035	XI	14	0	0	1·9
6. Suipestifer	„	1710	V, VI	11	0	0	1·5
7. *Staphylococcus aureus*	...	...	...	13	0	0	1·7
...	...	Control mice		20	0	0	1·7

* According to White (1926).
† Excluding one animal killed after surviving 71 days.

A study of Table VI leads to the following conclusions:

(*a*) Although inoculation has not succeeded in evoking solid immunity, its influence on the course of the disease is marked when the vaccine contains somatic antigen factors homologous to those in the infecting organism. In such cases (see vaccines 1 to 4) the average length of life was 6·5 to 7·9 days, as compared with 1·5 to 1·9 days for the mice either uninoculated or inoculated with antigenically unrelated vaccines (see controls and vaccines 5 to 7).

(*b*) The presence of H antigen in the vaccine does not increase its efficacy; vaccines 1 and 2 which contain Gärtner H antigen are no better than vaccines 3 and 4 which do not.

(*c*) *S. typhi* (Vaccine 4) protects as well as does *S. enteritidis*, Gärtner (vaccines 1 to 3), and the only antigen these organisms have in common is the somatic one. Similiar cross-protection in the case of human beings was reported by Herzog and Schiff (1922); they noted that during a Gärtner food-poisoning epidemic in a hospital typhoid convalescents remained significantly uninfected.

SUMMARY.

1. In the case of both *S. aertrycke* and *S. enteritidis*, Gärtner, infections in mice, it has been shown that the efficacy of prophylactic inoculation is dependent on the O or somatic antigen contained in the vaccine.

2. The presence or absence of H or flagellar antigen does not influence the prophylactic value of such vaccines.

3. The somatic antigen may for vaccine purposes be derived from a bacterial type alien to the infecting one, *e.g. S. paratyphi B* vaccine for protection against *S. aertrycke*, and *S. typhi* vaccine for protection against *S. enteritidis*, Gärtner, so long as the somatic antigen is in each case identical.

4. The immunogenic value of somatic antigen, as tested in the case of *S. aertrycke*, survives heating to 100° C. for ¼ hour, but is considerably lowered after 2 hours at that temperature.

5. After 9 months in the cold room paratyphoid B vaccine plus 0·5% phenol showed no deterioration in its prophylactic value.

6. The somatic antibody titre of an inoculated mouse may serve to some extent as index of its immunity, but resistance to infection is possible for mice without appreciable amounts of circulating O agglutinins demonstrable by *in vitro* tests.

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IMMUNIZATION OF THE HORSE WITH THE VIRUS OF POLIOMYELITIS AND THE PRODUCTION OF AN ANTIVIRAL SERUM.

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SERUM therapy in poliomyelitis has not hitherto aquired any universally recognized status. The main reason for this is that only the use of human convalescent serum has been generally accepted as of value (Netter, Gendron and Touraine, 1911; Amoss and Chesney, 1917; Davide, 1928; Macnamara, 1928; Aycock, Luther and Kramer, 1929, etc.). Such serum cannot always be readily obtained, and even when available the quantity at hand is usually insufficient for general application on a large scale. There is the further difficulty that except in times of epidemic, it is not as a rule possible to interfere early enough to secure any mitigation of the virus attack on the central nervous system.

Sera prepared artificially by the inoculation of horses and sheep with various antigens, have been exploited. Flexner (1912) stated that attempts to produce an antiviral serum in the horse had been disappointing, but that intramuscular injections of the virus offered more promise. Rosenow (1917) and Nuzum (1917), on the assumption that streptococci were ætiologically connected with the disease, prepared independently an antistreptococcal serum. This serum, however, has not been shown to have any definite neutralizing action on the virus (Amoss and Eberson, 1918[1], Stewart and Haselbauer, 1928). Pettit (1918) prepared a serum by intravenous inoculation of the sheep with cord emulsions. This process was extended to horses, and the resulting serum employed therapeutically with alleged good results (Jonesco-Mihaesti, 1928; Marinesco, etc., 1929). Recently Stewart and Haselbauer (1928) have found that this serum did not neutralize the virus when inoculated in mixture. Both human and monkey convalescent serum possess this property, and it has been utilized in testing the value of these sera for therapeutic purposes. Neustaedter and Banzhaf (1917), employing the intramuscular route, inoculated increasing doses of virus filtrate which they had trypsinized with the object of liberating endotoxin, and showed that the serum yielded had neutralizing value. Since then very little progress has been made, and as recently as 1928 Flexner and Stewart stated that no other experimental animal than the monkey had shown itself susceptible to inoculations of the virus, or had developed specific antibodies on repeated injections of virus-containing nervous tissues.

Thus, although many sera prepared in various ways have been and are being used therapeutically, only scanty evidence of specific action on the virus has been supplied. Statistical evidence of the value of serum treatment is

unfortunately of no great importance, owing to the varying severity of the disease in different epidemics and the difficulty of diagnosis in the preparalytic stages when treatment is most hopeful.

The following work deals with the successful immunization of a horse by the intramuscular route, using repeated and increasing doses of virus.

TECHNIQUE.

Immunization.—The virus emulsions were all prepared from the central nervous system of monkeys which had become prostrate following intracerebral inoculation of the very powerful "Fl. M." strain. As far as possible cord was employed, but owing to the large amounts required for the later inoculations, brain substance obtained from the region of the Rolandic fissures had to be used. The reason for selecting this site is explained in a current paper (Fairbrother and Hurst, 1930). The emulsions, after standing overnight at + 4° C., were passed through Berkefeld "N" filters, which permit the passage of this strain with great facility. Sufficient material was prepared at one time for each series of injections, and during the interval it was kept at 4° C.

The scheme of the inoculations, performed by Dr. W. T. J. Morgan at Elstree, together with the times of bleeding is shown in Table I. They were all given into the muscles of the fore-part of the animal.

TABLE I.—*Scheme of Immunization of Horse.*

9 . 5 . 29	Horse bled; normal serum.
24 . 5 . 29 27 . 5 . 29 30 . 5 . 29	50 c.c. of 0·5% strength.
6 . 6 . 29* 10 . 6 . 29	75 „ 1% „
13 . 6 . 29*	
17 . 6 . 29* 20 . 6 . 29* 24 . 6 . 29 27 . 6 . 29	100 „ 5% „
1 . 7 . 29	First bleeding.
4 . 7 . 29 8 . 7 . 29 11 . 7 . 29*	150 c.c. of 10% strength.
15 . 7 . 29	Second bleeding.
18 . 7 . 29 22 . 7 . 29 25 . 7 . 29	200 c.c. of 20% strength.
1 . 8 . 29	Third bleeding.

* = inoculation of tetanus toxoid in small doses.

The following points are worthy of mention: The animal received 15 separate injections over a period of some 2 months. Each inoculation was

given at an interval of 3 or 4 days, with 7 days between each series. During the earlier period a few small doses of tetanus toxoid were added; this was simply a routine procedure. During the whole course no definite sign of reaction in the horse, as determined by examination of the pulse-rate and rectal temperature, was observed. In order to obtain representative samples of serum for comparative purposes, blood was collected before, during and after the immunization, and the serum kept at 4° C. until required for the neutralization tests.

Neutralization tests.—These were performed in the following manner: The various amounts of serum and virus emulsion, with addition of saline to a total volume of 1 c.c., were well mixed in small sterile tubes, placed at 37° C. for 1½–2½ hours and left overnight at 4° C. After thorough mixing, 0·5 c.c. of the contents of each tube was inoculated intracerebrally under ether anæsthesia into separate monkeys.

In comparative work such as this, it is a matter of the greatest importance that the test strain of virus should be of uniform and high virulence. It is well known that some strains are very weak and by no means infect with regularity; the use of such a strain in the above test would be futile. The strain employed in this work (viz. "Fl. M."), however, was one of great potency, and as in some of the quantitative tests complete neutralization was not obtained, some details of its activity may be appended. The results following intracerebral inoculation of varying doses of a 5% emulsion are given in Table II.

TABLE II.

Dose.	Number of animals used.	Average incubation period.
0·5 c.c.	13	5·5 days
0·3 ,,	4	4·25 ,,
0·2 ,,	1	5 ,,
0·1 ,,	8	4·8 ,,
0·003 ,,	2	7 ,,
0·0005 ,,	1	0 ,,

0 = absence of infection.

It is seen that the strain is remarkably active and consistent. In doses similar to those employed in the neutralization tests, the incubation period, *i.e.* the time before the appearance of definite muscular weakness, was approximately 5 days. Recovery never occurred, death following in some 3 to 4 days after the onset of symptoms. It will be observed that the minimal infecting dose is roughly between 0·0005 c.c. and 0·003 c.c., *i.e.* the dose employed throughout in the tests is at least 100 times the minimal infecting dose. This is a very severe test, as the virus mixture is inoculated in all cases directly into the nervous substance of the brain.

RESULTS.

First Neutralization Test.

In order to titrate the various samples of serum, the amount of virus emulsion was kept constant while the quantity of serum was varied; 0·4 c.c. of a 5%

virus emulsion was mixed with 0·6 c.c. or 0·4 c.c. of the different samples of serum; in the latter case 0·2 c.c. of physiological saline was added. As an additional control 0·4 c.c. of the virus emulsion was mixed with 0·6 c.c. saline. The treatment described above was then carried out. The results are shown in Table III.

TABLE III.

Date of Test, 6 . 8 . 29.

Serum. Date of collection.	Amount.	Monkey.	Result.	Histology.*
...	0·6 c.c. saline	M. 124	Paralysis (5th day). Death	Poliomyelitis.
Normal . 9 . 5 . 29	0·6 „ serum	M. 116	„ (7th „). „	„
	0·4 „ „	M. 117	„ (5th „). „	„
1st bleeding 1 . 7 . 29	0·6 „ „	M. 118	No paralysis; death (19th day)	No poliomyelitis.
	0·4 „ „	M. 119	0	...
2nd „ 15 . 7 . 29	0·6 „ „	M. 120	0	...
	0·4 „ „	M. 121	No paralysis; death (16th day)	No poliomyelitis.
3rd „ 1 . 8 . 29	0·6 „ „	M. 122	„ „ (12th „)	Modified poliomyelitis.
	0·4 „ „	M. 123	„ „ (27th „)	No poliomyelitis.

0 = no infection. Death was frequently due to chloroforming when *in extremis*.

* Histological examination was performed on each case throughout this work by my colleague, Dr. E. W. Hurst.

This experiment was unfortunate in that the monkeys employed were far from satisfactory; 3 died from an intestinal infection, the exact nature of which was not ascertained, but similar cases have been encountered not infrequently, and from these, members of the Flexner dysentery group were isolated. Nevertheless, several interesting facts are shown:

(1) The normal serum, like the saline, had no effect on the virus, the incubation period being within the usual limits (*vide* Table II).

(2) Two animals, Ms. 119 and 120, remained perfectly well. Thus both the sera of the first and second bleeding had definitely acquired some antiviral property as a result of the injections. Three others, Ms. 118, 121 and 123, died probably from a dysentery infection and showed no trace of poliomyelitis, either clinically or histologically. Death occurred much later than in unmodified poliomyelitis, and as in the case of the sera of the first and second bleedings (Ms. 119 and 120) neutralization had been obtained in one case with a smaller amount of serum, it seems justifiable to exclude poliomyelitis as the responsible factor. One other animal, M. 122, died following marked diarrhœa with some leg weakness. Histological examination showed some nerve-cell destruction in the cord, but this was very slight compared with the usual histological picture at death for this particular strain (Hurst, 1929). Thus it is possible that here also the intestinal condition was the important factor in the death of the animal.

Evidence is thus adduced that the serum obtained during immunization has caused either a complete neutralization or some attenuation of the action of the virus; in the latter case the result was prolongation of the incubation

period with alteration of the usual clinical and histological picture. It seems highly probable, however, that the former action—complete neutralization—had occurred, the other phenomena, diarrhœa, death, etc., being the result of a secondary infection.

Second Neutralization Test.

A further attempt to titrate the various samples of serum was made. The virus amount in all cases was 0·6 c.c., while the serum was added in much smaller amounts, 0·1, 0·2 and 0·2 c.c.—saline being again added where required. The results are shown in Table IV.

TABLE IV.

Date of Test, 2 . 10 . 29.

Serum.		Amount.	Monkey.	Result.	
...	.	0·4 c.c. saline	M. 138	Paralysis (4th day).	Death.
Normal	9.5.29	0·4 „ serum	M. 137	„ (5th „).	„
		0·2 „ „	M. 136	„ (6th „).	„
		0·1 „ „	M. 129	„ (7th „).	„
1st bleeding	1.7.29	0·2 „ „	M. 128	„ (6th „).	„
		0·4 „ „	M. 127	„ (10th „).	„
		0·1 „ „	M. 130	„ (5th „).	„
2nd „	15.7.29	0·2 „ „	M. 131	0	
		0·4 „ „	M. 132	Paralysis (11th day).	Death.
		0·1 „ „	M. 133	„ (9th „).	„
3rd „	1.8.29	0·2 „ „	M. 134	„ (17th „).	„
		0·4 „ „	M. 135	„ (22nd „).	„

Neutralization was obtained in only one instance, M. 131. Examination of the incubation periods of the different animals yielded, however, valuable information of the comparative merits of the sera. The following points are worthy of note :

(1) The normal serum again had no effect.

(2) The first bleeding serum gave slight lengthening of the incubation period when 0·4 c.c. was used, otherwise there was no variation from the normal.

(3) Second bleeding serum gave a rather more marked action when 0·2 and 0·4 c.c. were used. The former neutralized the virus, while the latter amount gave a prolongation of the incubation period (11 days). M. 131, which remained uninfected, was suffering from early pulmonary tuberculosis, which might have in some way influenced the result.

(4) Third bleeding serum very definitely led to prolongation of the incubation period, which was increased as the larger doses were employed. Complete neutralization did not occur. Mention might be made that from the beginning M. 135 was in an extremely emaciated condition.

An examination of the results of these two experiments yields several interesting facts. In the first place the serum has been found to possess distinct power to neutralize the action of the virus; moreover, this power increased *pari passu* with the strength of the inoculations in the horse. In other words, it is found that the third bleeding serum, obtained shortly after the final injection, has a more powerful action than those collected at earlier

stages. Also it is seen that when the amount of serum is equal to or greater than that of the virus emulsion, complete neutralization is procured. When, however, the ratio is reversed, and the amount of virus emulsion is greater than that of serum, some virus escapes the action of the serum with proportionate prolongation of the incubation period, but once the disease commences, its course is as usual—rapid prostration leading to a fatal issue. That the serum did not possess any antiviral properties before the inoculations commenced is shown in an unequivocal manner.

Third Neutralization Test.

The previous experiments have all been performed under conditions definitely favourable to the virus, as the amount of virus emulsion, which itself was many times the minimal infecting dose, usually closely approximated to that of the serum. Thus the test was essentially very severe, yet it has been seen that when the serum was equal to or greater than the amount of virus emulsion, infection on subsequent inoculation did not occur. In the following test use was made of proportions of serum and virus emulsion similar to those employed in previous comparative work (Stewart and Heselbauer (*loc. cit.*); Stewart and Rhoads, 1929); the amount of virus emulsion was here decidedly less than that of the serum. The results are given in Table V.

TABLE V.

Date of Test, 19.11.29.

Serum.	Mixture.	Monkey.	Result.
Normal . 9.5.29	0·1 c.c. virus + 0·9 c.c. serum	M. 160	Paralysis (8th day). Death.
2nd bleeding 15.7.29	0·2 „ „ + 0·8 „ „	M. 162	0
3rd „ 1.8.29	0·2 „ „ + 0·8 „ „	M. 163	0
	0·2 „ „ + 0·4 „ „ + 0·4 c.c. NaCl	M. 164	0
	0·2 „ „ + 0·2 „ „ + 0·6 „ „	M. 165	0
	0·2 „ „ + 0·1 „ „ + 0·7 „ „	M. 166	0

Confirmation of the previous results has been obtained in a very striking manner. The normal serum had little, if any, influence on the virus, whereas the immune serum in smaller amounts neutralized the action of double the quantity of virus emulsion. The third bleeding serum again exerted well-marked antiviral properties; in the case of M. 166, in which the proportion of serum to virus was 1 to 2, the animal remained quite well. This result may be compared with that in M. 135, where, with a ratio of 6 of virus to 4 of serum, the animal became infected after a very prolonged incubation period of 22 days. Here, however, the animal was always thin and weak, with consequent low natural resistance. Thus it is seen that when the amount of virus is not excessive the serum of the immunized horse completely neutralizes its action in a manner apparently comparable with that of human and monkey convalescent serum.

Fourth Neutralization Test.

In order to serve as a guide to the relative potency of the horse-serum, several samples of convalescent serum, human and monkey, were tested in a

similar manner. The supply of these was unfortunately limited, and is possibly not very representative of the action of convalescent serum as a whole. The results are given in Table VI.

TABLE VI.

Serum. Origin.	Serum. Time after infection.	Mixture (dose 0·5 c.c.).	Monkey.	Result.
D—(30 years)	3 months	0·2 c.c. virus + 0·8 c.c. serum	M. 107	0
		0·6 „ „ + 0·2 c.c. serum + 0·2 c.c. NaCl.	M. 148	Paralysis (7th day). Death.
		0·6 „ „ + 0·1 „ „ + 0·3 „ „	M. 150	Paralysis (8th day). Death.
St—(14 „)	14 years	0·5 „ „ + 0·5 „ „	M. 140	0
A—(9 „)	1 month	0·2 „ „ + 0·8 „ „	M. 105	0
		0·6 „ „ + 0·2 „ „ + 0·2 „ „	M. 158	Paralysis (14th day). Death.
Monkey M. 10	30 days	0·2 „ „ + 0·8 „ „	M. 106	0

The results are analogous to those obtained with the horse-serum. When the proportion of serum to virus was greater than or equal to unity, protection occurred, but when the virus amount exceeded that of the serum, infection took place after a lengthened incubation period. It does not seem fair to make a definite comparison of the two series of results, but that the action of the third bleeding serum on the virus was at least equal to that of the serum of some convalescents seems quite definite.

Fifth Neutralization Test.

A further bleeding (fourth) of the horse was performed on 26 . 9 . 29, and some two months after the last inoculation of virus; during this time the horse had been resting. Neutralization tests were performed with this sample and also with the serum of the third bleeding (1 . 8 . 29); in all cases the ratio of serum to virus was equal to or greater than unity. The results are shown in Table VII.

TABLE VII.

Date of Test, 21 . 10 . 29.

Serum.	Mixture (dose 0·5 c.c.).	Monkey.	Result.	Histology.
3rd bleeding 1.8.29	0·2 c.c. virus + 0·8 c.c. serum	M. 114	0	...
	0·4 „ „ + 0·6 „ „	M. 154	0	...
	0·5 „ „ + 0·5 „ „	M. 152	No paralysis; death (20th day)	No poliomyelitis.
4th bleeding 26.9.29	0·4 „ „ + 0·6 „ „	M. 156	Paralysis (10th day). Death	Poliomyelitis.
	0·5 „ „ + 0·5 „ „	M. 155	No paralysis; death (7th day)	No poliomyelitis.

Once again the results were deprived of their full significance by the presence of an intestinal infection in two of the animals, Ms. 152 and 155. Nevertheless, several interesting facts are apparent. The third bleeding serum once again exhibited definite antiviral properties—it seems highly probable that M. 152 would have remained entirely free from poliomyelitis had the intestinal infection not supervened. The reasons for such an assumption are that the animal lived for 20 days after the injection, and never showed any signs, clinical or histological, of poliomyelitis. Moreover, as illustrated by the experience with Ms. 114 and 154, the serum was found to contain antiviral bodies. It would appear, however, that during the resting phase of 2 months the antiviral content had decreased, for M. 156 became infected with poliomyelitis after a moderate lengthening of the incubation period—10 days (vide Table II); the proportion of serum to virus was here greater than unity, and under such circumstances the serum of the earlier bleedings, taken during the course of immunization, afforded complete neutralization. The result in the case of M. 155 was nullified by the death of the animal, for reasons given, in 7 days. The probability of poliomyelitis occurring subsequently if the animal had lived is great, as M. 156, which received a relatively larger amount of serum than virus, became infected after a prolonged incubation period.

Curative Tests.

The previous experiments have been performed under purely artificial conditions, and can by no means be taken as an accurate representation of the reaction taking place *in vivo*. It was decided to adopt some curative measure comparable with the application of serum therapy in the human disease. With this intention three animals were inoculated intracerebrally with 0·02 c.c. of a 5% emulsion; subsequently at different intervals the third bleeding serum, 1 . 8 . 29, was administered by various routes—intravenous, intrathecal and intramuscular, alone or in combination. The results are given in Table VIII.

TABLE VIII.

Date of Test, 21 . 10 . 29.

Animal.	Time after virus.	Route.	Result.	Histology.
M. 153	6 hours	Intravenous . 40 c.c. Intrathecal . 1 „	Died (2 days)	No poliomyelitis.
M. 142	1 day	„ . 1 „ + intravenous . 20 c.c.	Died (9 days)	Ditto.
	2 days	Intravenous . 10 „		
	3 „	Intramuscular 12 „		
	5 „	„ 20 „		
M. 146	1 day	Intrathecal . 1 „ + intravenous . 18 c.c. + intramuscular 12 „	Died (10 days)	Poliomyelitis present.
	2 days	Intramuscular 10 „		
	3 „	„ 12 „		
	5 „	„ 14 „		

Nothing of a definite nature was demonstrated. One animal, M. 153, never recovered after the intravenous injection, but died of right heart failure in 2 days. Another, M. 146, died in 10 days, having shown definite leg weakness and marked diarrhœa. Histological examination of the cord yielded definite signs of poliomyelitis. The other, M. 142, died of an intestinal infection

after 9 days. No sign of poliomyelitis, clinically or histologically, was observed, and in this case it is possible that the administration of the serum had checked the progress of the disease. However, the practical value of this experiment is almost *nil*, owing to the occurrence of the secondary infection. It must be stated that serum therapy of monkeys inoculated intracerebrally with a potent virus is obviously an entirely different proposition from similar treatment in the human condition. In the former case the mortality-rate is approximately 100% as against 10–20% in man; moreover, the virus is implanted directly into the nervous substance, which sustains a certain degree of trauma. An attempt had previously been made to modify the action of the virus introduced intracerebrally into 2 monkeys by the simultaneous intrathecal and intravenous administration of human convalescent serum after 24 hours and 3 days respectively. The course of the disease was not altered in the least; the incubation period in both cases was the usual 5 days. Thus it is seen that curative tests in monkeys present many difficulties, success being exceptional and only obtained by the early inoculation of large amounts of serum, which, however, tends to excite latent infections.

DISCUSSION.

Evidence has been brought forward which suggests that the intramuscular immunization of a horse by increasing doses of poliomyelitis virus has led to the development of antiviral bodies in the serum. In order to establish the value of this serum it would be well to examine the test on which this evidence is mainly based. This was the so-called neutralization test, in which the virus and serum were allowed to remain in contact *in vitro*, after which an aliquot amount (0·5 c.c. usually) was inoculated intracerebrally into susceptible monkeys (*M. rhesus*).

This test has been utilized extensively in the study of poliomyelitis. Levaditi and Landsteiner (1910), Römer (1911), and Flexner and Lewis (1910[1]), found that when serum of recovered monkeys was allowed to act on the virus for some hours *in vitro*, the action of the virus on subsequent intracerebral inoculation of the mixture was neutralized; normal monkey serum did not possess this property. Netter and Levaditi (1910) showed that serum of individuals recovered from the disease had this action, as also had the serum from abortive cases. Anderson and Frost (1911) confirmed this latter finding, and found that serum from apparently normal people also contained antiviral bodies. These were shown to be less active than in the abortive cases by a partial titration with virus emulsion of both 5% and 1% strengths. It is highly probable, however, that these apparently normal people had acquired some active immunity without displaying any clinical manifestation of the disease. Aycock and Kagan (1927), and, later, Stewart and Rhoads (1929) have immunized monkeys by repeated intradermal inoculations of small doses of the virus without causing any detectable clinical disturbance. The monkeys were found to resist subsequent intracerebral inoculation of the virus while their sera contained the neutralizing substances. The latter test—that of neutralization—was considered by Stewart and Rhoads a more delicate test of immunity-response than intracerebral inoculation. It has thus been demonstrated by a large number of independent workers that these antiviral bodies

are present only with regularity in convalescent human and monkey sera, but that they can, however, be produced by active cutaneous immunization of the monkey. The only other serum which has been found to possess this property is that of sheep; its presence to any degree, however, is not consistent (Flexner and Lewis, 1910[2]; Stewart and Haselbauer, *loc. cit.*). The test may, therefore, claim a high degree of specificity, and it has been employed repeatedly to evaluate sera prepared artificially (Amoss and Eberson, 1918[3]; Stewart and Haselbauer). In all cases it must be noted that the amount of virus emulsion or filtrate at a 5% strength was equal to or less than that of the undiluted serum; in some cases the proportion of serum to virus filtrate was as much as 9 to 3 and 10 to 1. In fact, Rhoads (1930) employed a mixture of 0·99 c.c. serum and 0·01 c.c. Berkefeld "N" filtrate of 5% emulsion of a very active strain. A similar test has been employed successfully in many other virus diseases. Thus from the accumulation of evidence the use of the neutralization test seems justifiable, and the results obtained by its use must have a certain significance.

The exact *modus operandi* of the reaction has not been ascertained. It would appear that the immune serum does not destroy the virus during contact *in vitro*, as Olitsky, Rhoads and Long (1929) have been able to recover, by cataphoresis, the active virus from a serum-virus mixture, which was ineffective on intracerebral inoculation. As in the case of the herpes virus (Zinsser and Tang, 1929), the reaction does not require the presence of complement. Flexner and Amoss (1913) have shown that inactivated serum is just as effective as unheated serum, and this observation has been confirmed by us. Evidence is available suggesting that action by the serum on the virus during contact *in vitro* takes place only slowly. Leiner and v. Weisner (1910) found that after 4 hours' contact at room temperature, inoculation of the serum-virus mixture resulted in a light paresis, while inoculation after 6 hours' contact yielded a negative result. It was found, moreover, during the present work that inoculation of equal quantities of third bleeding serum and virus immediately after mixing resulted in infection after an incubation period of the usual 5 days. A like mixture, similarly inoculated, after the usual period of contact *in vitro* was not infective. In this connection mention should be made of a recent observation that changes indicating any local specific action by the poliomyelitis virus at the site of inoculation are not found to any marked degree, the main attack being on the nerve-cells of the cord (Fairbrother and Hurst, *loc. cit.*). In the case of intradermal inoculations of vaccinia virus the action is mainly local, so that the two reactions are not strictly comparable. It would seem, therefore, that the virus slowly becomes sensitized by the action of the immune serum, so that on subsequent inoculation it no longer has the power to infect otherwise susceptible animals. Normal animal serum has not this property, which is apparently acquired and specific; its presence in normal sheep-serum is the one known exception.

In view of the above facts it can hardly be argued that the method by which the serum was tested was inadequate, more especially as in all cases the test virus was one of the highest virulence, and the proportion of serum to virus was subjected to many changes and never greatly favoured the former constituent. It must, however, be admitted that tests of a curative nature

would have been more valuable had their application not presented so many difficulties.

The general trend of the results in this work is very striking. It has been shown that when the ratio of serum to virus was equal to or greater than one, the serum collected during the course of immunization prevented subsequent infection by the virus in almost all cases, the one exception being M. 122, in which other factors doubtless played an important *rôle*. When this ratio was reversed and the virus exceeded the serum, infection occurred after a relative prolongation of the incubation period. The partial titrations performed by this procedure suggested that the serum obtained at the end of the course of injections, the third bleeding serum, was richer in antiviral substances than the samples recovered earlier. It has also been shown that the neutralizing power of the serum tended to diminish rather rapidly when the injections were discontinued. The normal horse-serum, obtained before the injections commenced, was never found to have the slightest influence on the virus.

Another interesting fact which should be mentioned, but not emphasized, as only a few samples of human convalescent sera were tested, was that the results obtained from these few tests suggest that their antiviral content was no greater than that of the third bleeding serum of the horse.

In all these tests consideration should be made of the fact that the test dose of virus was at least 100 times the minimal infecting dose on intracerebral inoculation. Thus, in all cases where neutralization took place, the sera must have possessed marked activity.

Reasons for the failure of other workers to obtain a satisfactory serum do not appear obvious. The results given above, however, confirm the findings in the small series of neutralization tests recorded by Neustaedter and Banzhaf (*loc. cit.*), obtained by the use of a serum prepared in a similar but not identical manner. Attention should be drawn to the fact that enormous doses of virus were given by the intramuscular route, and that brain substance was employed as little as possible in the preparation of the emulsion. It may be, however, that the horse employed was particularly responsive to the virus inoculations, as it is well known that horses differ considerably in their power to produce antitoxin following toxin inoculation. The findings here recorded serve to contravene the statement that only in animals susceptible to a certain virus is it possible by immunization to obtain neutralizing substances in the serum against that virus. In the hope of elucidating further some of these points another horse is being inoculated with similar doses but by the intravenous route.

CONCLUSIONS AND SUMMARY.

A horse has been successfully immunized by repeated intramuscular inoculations of increasing doses of a potent virus.

The resulting serum has been found to contain antiviral properties as shown by the neutralization test.

The application of curative tests was not successful for reasons given.

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THE HÆMOLYTIC PROPERTIES OF CHOLERA, PARA-CHOLERA AND ALLIED VIBRIOS, WITH SPECIAL REFERENCE TO THEIR EFFECT ON BLOOD MEDIA.

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The question of the hæmolytic properties of *V. choleræ* and allied vibrios gained particular prominence in 1905, when Gottschlich isolated the El Tor vibrios. Since then much work has been done in an attempt to differentiate *V. choleræ* from other vibrios by the ability of the latter to hæmolyse blood.

The subject has recently been reviewed by Mackie (1929), who shows that a good deal of confusion and uncertainty exists in regard to the question of the hæmolytic properties of vibrios. Much of this uncertainty has arisen from the variation in the methods used. In testing hæmolysis there is an essential difference between adding a fully grown fluid culture to a blood suspension and by actually growing the particular organism in a solid medium containing blood.

In the experiments recorded in this paper, the "hæmolytic" effects of cholera, paracholera and allied vibrios have been re-studied, and special attention has been paid to the property of transforming hæmoglobin into colourless derivatives (see Kovács, 1926). The tests have been carried out with the blood of various animal species in parallel.

Strains of cholera, paracholera, El Tor and "cholera-like" vibrios were obtained from the National Collection of Type Cultures, from the Bacteriology Department, Edinburgh University, and from Major Maitra, I.M.S., to whom I am indebted for sending me freshly isolated strains of vibrios isolated from cases of cholera, cholera carriers and from water, with notes on the sources from which they were obtained.

The general biological characters of these strains were also studied.

Two anticholera sera (prepared from different strains of *V. choleræ*) were used for serological tests.

EXPERIMENTS.

The hæmolytic action of various strains of cholera, paracholera and "cholera-like" vibrios was tested by adding cultures grown in neutral peptone-water to suspensions of ox, rabbit, goat, sheep, horse, rat, guinea-pig and human blood. A standard suspension of 3% washed red cells in 0·85% saline was used, and hæmolysis was tested by adding varying quantities of the culture to a series of tubes containing 1 c.c. of the blood suspension. The tubes were incubated for 4 hours at 37° C., and readings were then taken. A number of illustrative results are shown in Table I.

TABLE I.—*Hæmolysis Produced by Peptone Water Cultures of Cholera, Paracholera and Cholera-like Vibrios.*

Organism.	Blood suspension.							
	Ox.	Rabbit.	Goat.	Sheep.	Horse.	Rat.	Guinea-pig.	Man.
V. choleræ "Bombay"	0	0	0	0	0	0	0	0
V. choleræ "Singapore"	0	tr.	0	0	0	0	0	0
V. paracholeræ A (Mackie and Storer)	+++	+++	+++	+++	+++	+++	+++	+++
,, *B* ,, ,,	+++	+++	+++	+++	+++	+++	+++	+++
,, "Belah" (Mackie)	+++	+++	++	++	++	+++	+++	++
,, "K" (Mackie)	+++	+++	++	+++	++	+++	+++	++
V. Finkler and Prior	0	0	0	0	0	0	0	0
V. El Tor (Gottschlich)	+++	+++	+++	+++	+++	+++	+++	+++
V. Metchnikovi	+++	+++	+	tr.	+	+	tr.	tr.

In this and subsequent tables the degree of hæmolysis is indicated by + signs; tr. = trace of hæmolysis; 0 signifies absence of hæmolysis.

As shown in Table I, vibrios corresponding to the classical type of *V. choleræ* are non-hæmolytic when tested by adding fluid cultures to a blood suspension, whereas the paracholera and El Tor vibrios are markedly hæmolytic. The lysin produced by *V. Metchnikovi* varies in its action on the red cells of different species, ox and rabbit red cells being more markedly affected than those of the other animals tested. Certain other vibrios showed similar effects and were markedly lytic to ox and rabbit blood, though their action on goat and sheep blood was slight or absent. This is illustrated in Table II.

TABLE II.—*Showing Variation in Resistance of Red Cells of Different Species of Animals to the Hæmolysins of Certain Vibrios.*

Vibrio.	Blood suspension.			
	Ox.	Rabbit.	Goat.	Sheep.
4154	+++	+++	+	0
18	+++	++	0	0
2913	++	++	0	+

TABLE III.

Vibrio.	Hæmolysis produced by fluid culture added to blood-suspension.				Apparent hæmolysis or clearing by 24 hours' growth on blood-agar.			
	Ox.	Rabbit.	Goat.	Sheep.	Ox.	Rabbit.	Goat.	Sheep.
V. paracholeræ A	+++	+++	+++	+++	+++	+++	+++	+++
,, *B*	+++	+++	+++	+++	+++	+++	+++	+++
,, "K"	+++	+++	+++	+++	+++	+++	+++	+++
,, "Belah"	+++	+++	++	++	+++	+++	+++	+++
V. El Tor	+++	+++	+++	+++	+++	+++	+++	+++
10	+++	+++	+++	+++	+++	+++	+++	+++
C.	+++	+++	+++	+++	+++	+++	+++	+++
6	+++	+++	+++	+++	+++	+++	+++	+++
2075	+++	+++	+++	+++	++	+++	+++	+++
V. choleræ (Bombay)	0	0	0	0	0	0	0	0
,, (Singapore)	0	tr.	0	0	0	0	0	0
251 (Liston)	0	tr.	0	0	0	0	0	0
3077	0	tr.	0	0	0	tr.	0	0
3134	0	tr.	0	0	0	tr.	0	0
1293	0	0	0	0	0	tr.	0	0
1292	0	0	0	0	0	0	0	0
1358	0	0	0	0	0	0	0	0
6178	+++	+++	+++	+++	+++	+++	+++	+++
8465	++	++	++	++	+++	+++	+++	+++
7584	++	++	++	++	+++	+++	+++	+++
7656	+++	+++	+++	+++	+++	+++	+++	+++
6482	+++	+++	+++	+++	+++	+++	+++	+++
8591	+++	+++	+++	+++	+++	+++	+++	+++
9322	0	0	0	0	++	++	++	++
5716A	0	0	0	0	++	++	++	++

The strain 4154 corresponded in serological character to the classical cholera strain. Strains 18 and 2913 resembled *V. choleræ* in general characters, but were not agglutinated by an antiserum for a typical cholera strain.

It is apparent from such results that goat and sheep red cells show greater resistance to vibrio hæmolysins than ox or rabbit cells. As the result of a large number of experiments it appeared that the red cells of the goat are most resistant and those of the rabbit most susceptible (in the series tested) to the action of vibrio hæmolysins. Ponder and McLachlan (1927) demonstrated that the lysin of the El Tor vibrio differed from those produced by other bacteria in that the blood corpuscles of certain animals were less susceptible to its action than those of other species; thus, human red cells showed considerably greater resistance than rabbit corpuscles. They did not use goat blood in their investigation.

Those strains of vibrios which lysed blood suspensions were also "hæmolytic" when grown on agar to which 5% red cells had been added. Certain vibrios, however, which were unable to lyse blood in suspension, were apparently lytic when grown on 5% blood-agar (*vide* Table III).

It was found that, in general, parallel results were obtained if, in the test on blood-agar, readings were taken in 24 hours; but 2 strains, V. 9322 and V. 5716A, "cleared" blood-agar in 24 hours, though no lysis occurred when culture was added to blood suspensions. The clearing, however, of blood-agar by V. 9322 and V. 5716A in 24 hours was not so marked as that produced by the El Tor or paracholera vibrios in the same time.

The relationship of the hæmolytic properties of the vibrios studied to their serological characteristics is of interest, and is shown in Table IV.

It appears from the above table that with the exception of strain C, all the hæmolytic strains of "non-agglutinating" vibrios were from active and convalescent cases of choleraic disease. Strain C was obtained 48 hours after inoculation of a "vibrio-free" tank with dejecta from a case of acute cholera.

The vibrios in the series examined which proved to be non-hæmolytic and did not react with an antiserum for *V. choleræ* were recorded as having been isolated from convalescent cases of clinical cholera, from healthy carriers and from water-tanks. It will also be seen that several strains of vibrios with hæmolytic properties, isolated from cases of clinical cholera, were agglutinated by an antiserum to *V. choleræ*. This group included two El Tor strains.

In view of Kovác's observation (1926) referred to above, the various strains were tested by growing them on 5% heated blood-agar and 5% fresh blood-agar; parallel readings of the clearing produced on the respective media were made after incubation for 24 hours at 37° C. Ox blood was used. Heated blood-agar was prepared by adding 5% blood by volume to liquid agar at 55° C. The agar containing blood was then boiled for a few seconds and allowed to cool. On cooling it presented a "chocolate" appearance.

The strains 9322 and 5716A, which, as already shown, produced no lysis of blood in suspension, but which cleared blood-agar within 24 hours, cleared heated blood also in 24 hours (Table V). "Clearing" of blood-agar must therefore be distinguished from true "hæmolysis." Some strains are able to lyse blood when fluid culture is added to blood suspension, and also to produce clearing when grown on agar containing fresh or heated blood. Such strains

TABLE IV.—*Relation of Hæmolysis to Serological Characteristics.*

Vibrio.	Serological type.	Hæmolysis (fluid culture added to suspensions of ox and sheep blood).	Source.
V. choleræ (Bombay)	Agglutinated by an anticholera serum	0	Lab. strain, India.
,, (Singapore)	,, ,, ,,	0	N.C.T.C., Malaya.
251 Liston	,, ,, ,,	0	,, India.
253 ,,	,, ,, ,,	0	,, ,,
3134	,, ,, ,,	0	Fatal case of cholera, India.
A.	,, ,, ,,	0	Water-tank contaminated with fæces, India.
3021	,, ,, ,,	0	Fatal case of cholera, India.
9322	,, ,, ,,	0	Case of clinical cholera, India.
V. El Tor	,, ,, ,,	+++	Lab. strain, Mesopotamia.
,, 1908	,, ,, ,,	+++	N.C.T.C., Mesopotamia.
9307	,, ,, ,,	+++	Case of clinical cholera, India.
9350	,, ,, ,,	+++	,, ,, ,,
9309	,, ,, ,,	+++	,, ,, ,,
1293	Not agglutinated by an anticholera serum	0	Cholera convalescent, India.
1292	,, ,, ,,	0	,, ,, ,,
1358	,, ,, ,,	0	Water-tank, India.
4014	,, ,, ,,	0	,, ,,
1360	,, ,, ,,	0	Healthy cholera carrier, India.
V. Finkler and Prior	,, ,, ,,	0	N.C.T.C.
No. 2	,, ,, ,,	0	Lab. culture, India.
1368	,, ,, ,,	0	,, ,,
1347	,, ,, ,,	0	Cholera convalescent, India.
4190	,, ,, ,,	0	Water-tank, India.
4925	,, ,, ,,	0	Healthy carrier, India.
5716A	,, ,, ,,	0	Case of clinical cholera, India.
V. paracholeræ A	,, ,, ,,	+++	Lab. strain, Mesopotamia.
,, *B*	,, ,, ,,	+++	,, ,, ,,
,, "K"	,, ,, ,,	+++	,, ,, ,,
,, "Belah"	,, ,, ,,	+++	,, ,, ,,
10	,, ,, ,,	+++	Case of clinical cholera, India.
C.	,, ,, ,,	+++	,, ,, ,,
6	,, ,, ,,	+++	,, ,, ,,
2975	,, ,, ,,	+++	,, ,, ,,
8540	,, ,, ,,	+++	,, ,, ,,
7978	,, ,, ,,	+++	,, ,, ,,
8228	,, ,, ,,	+++	,, ,, ,,
8465	,, ,, ,,	+++	,, ,, ,,
8591	,, ,, ,,	+++	,, ,, ,,
6482	,, ,, ,,	+++	,, ,, ,,
7584	,, ,, ,,	+++	,, ,, ,,
7656	,, ,, ,,	+++	,, ,, ,,
7933	,, ,, ,,	+++	,, ,, ,,
4582	,, ,, ,,	+++	,, ,, ,,
6128	,, ,, ,,	+++	,, ,, ,,

N.C.T.C.=National Collection of Type Cultures, Lister Institute, London.

apparently yield a true hæmolysin, but are also capable of producing clear areas on heated blood media. That the two properties are independent is shown by the inability of the El Tor and paracholera vibrios to clear heated blood whilst producing active hæmolysis of blood in the tests with blood suspensions or blood-agar. The possession of true hæmolytic properties cannot be attributed to a vibrio unless it causes hæmolysis of blood suspensions.

TABLE V.

Vibrio.	Serological type.	Hæmolysis or "clearing."		Fluid culture added to blood suspension.
		Grown for 24 hours on agar containing—		
		Heated blood.	Fresh blood.	
V. choleræ (Bombay)	Agglutinated by an anti-cholera serum	0	0	0
253 Liston	,, ,, ,,	0	0	0
V. choleræ (Singapore)	,, ,, ,,	0	0	0
3021	,, ,, ,,	0	0	0
A	,, ,, ,,	0	0	0
3134	,, ,, ,,	0	0	0
9307	,, ,, ,,	+++	+++	+++
9350	,, ,, ,,	+++	+++	+++
C9309	,, ,, ,,	+++	++	+++
9322	,, ,, ,,	+++	+++	0
V. El Tor	,, ,, ,,	0	+++	+++
,, 1908	,, ,, ,,	0	+++	+++
4190	Not agglutinated by an anti-cholera serum	0	0	0
1293	,, ,, ,,	0	0	0
1358	,, ,, ,,	0	0	0
1292	,, ,, ,,	0	0	0
4014	,, ,, ,,	0	0	0
1368	,, ,, ,,	0	0	0
4935	,, ,, ,,	0	0	0
1292	,, ,, ,,	0	0	0
1360	,, ,, ,,	0	0	0
13	,, ,, ,,	0	0	0
No. 2	,, ,, ,,	0	0	0
1347	,, ,, ,,	0	0	0
V. paracholeræ "Belah"	,, ,, ,,	0	+++	+++
,, *A*	,, ,, ,,	0	+++	+++
,, *B*	,, ,, ,,	0	+++	+++
,, K	,, ,, ,,	0	+++	+++
7933	,, ,, ,,	0	+++	+++
8540	,, ,, ,,	+++	+++	+++
7978	,, ,, ,,	+++	+++	+++
8288	,, ,, ,,	+++	+++	+++
8465	,, ,, ,,	+++	+++	+++
8591	,, ,, ,,	+++	+++	+++
6482	,, ,, ,,	+++	+++	+++
7584	,, ,, ,,	+++	+++	+++
7656	,, ,, ,,	+++	+++	+++
5716A	,, ,, ,,	+++	++	0

The ability of some vibrios to produce clear areas around their growths when cultivated on heated blood-media is of considerable interest, and in an attempt to study the mechanism of this phenomenon, strains of hæmolytic vibrios which differed in their capacity to clear heated blood were grown on agar to which had been added 5% of crystalline hæmoglobin. The results of these experiments are illustrated in Table VI.

It will be noted that vibrios which produce zones of clearing on heated

TABLE VI.

Vibrio.	Blood-agar (10% sheep blood).	Agar containing 10% heated sheep blood.	Agar containing 5% hæmoglobin.
V. choleræ (Bombay) . . .	0	0	0
V. paracholeræ "K" . . .	+ + +	0	0
V. El Tor 2890	+ + +	0	0
V. paracholeræ A . . .	+ + +	0	0
" "Belah" . .	+ + +	0	0
" *B* . . .	+ + +	0	0
4582	+ + +	0	0
8591	+ + +	+ + +	+ + +
6482	+ + +	+ + +	+ + +
7656	+ + +	+ + +	+ + +
8228	+ + +	+ + +	+ + +
9322	+ + +	+ + +	+ + +

Number of + signs indicates degree of clearing in 24 hours.

TABLE VII.—*Strains Agglutinated by an Anti-cholera Serum.*

Group.	Source.				Number of strains.	Hæmolysis.	Clearing of heated blood.	Type.
	Clinical cholera.	Carriers.	Water.	Unknown.				
I	5 (3 Lab. strains)	0	1	5	11	−	−	*V. choleræ* (Koch).
II	3	0	5	19	27	+	+	..
III	2 (both Lab. strains)	0	0	0	2	+	−	El Tor.
IV	1	0	0	0	1	−	+	..
Total .	11	0	6	24	41	..	..	..

Strains not Agglutinated by an Anti-cholera Serum.

I	4	3	5	2	14	−	−	..
II	8	0	7	17	32	+	+	..
III	6 (4 Lab. strains)	0	4	8	18	+	−	Paracholera.
IV	1	0	0	0	1	−	+	..
Total	19	3	16	27	65	..	..	..

In the above table all strains except those designated "Lab. strains" were obtained from India. Of the "Lab. strains" two cholera strains were originally isolated in India, one in Singapore. The El Tor and paracholera strains were isolated in the Near East.

blood-agar produce similar clear areas when grown on agar containing 5% crystalline hæmoglobin. It would appear that the clearing effect of these vibrios on heated blood is due to their action on the hæmoglobin, *e.g.*, its conversion into colourless derivatives.

Spectroscopic examinations were made of the clear areas surrounding the colonies of different strains of vibrios on blood-agar and agar containing hæmoglobin. The clear zones on blood-agar produced by *V. paracholeræ A* and *B* and *V. El Tor* 2890 showed the characteristic absorption spectrum of oxy-hæmoglobin. On the other hand, when the clear areas on blood-agar surrounding the colonies of certain vibrios, *e. g.* Nos. 7656, 8228 and 9322, were examined, the absorption spectrum of alkali hæmatin was found. This was also the case when vibrios 7656, 8228 and 9322 were grown on heated blood-agar and agar containing 5% hæmoglobin.*

The function of the vibrios in liquefying coagulated serum was also examined. For the determination of the serum-liquefying power, tubes containing 2 c.c. of coagulated serum were inoculated by a straight stab, the inoculum being taken from an 18 hours' peptone-water culture. In all cases where the organisms produced clearing of heated blood-agar, some degree of liquefaction of serum was also found, but the extent of liquefaction was found to vary considerably with different strains of vibrios.

Thus, the hæmolytic vibrios which are agglutinated with an anticholera serum may be classified in two groups according to their action on heated blood-agar. One of these comprises the El Tor vibrios.

The paracholera vibrios isolated in Egypt and Palestine, although not agglutinated by an anticholera serum, resemble the El Tor vibrios in forming a true hæmolysin and do not produce clearing of heated blood-agar. It is of interest to note that the El Tor vibrios and the paracholera vibrios isolated by Mackie were obtained from choleraic cases in the Near East. Of the 97 Indian strains of vibrios examined, none corresponding to the El Tor vibrios in agglutination reactions, hæmolysis and absence of effect on heated blood were found, whereas 6 Indian strains corresponded to the paracholera vibrios.

The antigenic relationships of the strains which differed serologically from *V. choleræ* were examined. It was not considered practicable to prepare agglutinating sera for every strain, and anti-sera for certain selected strains were therefore used (*v. infra*). The reaction of the different strains with these sera in agglutination and agglutininin-absorption tests were considered sufficient to indicate whether or not there was antigenic similarity between the strain tested and that used for the preparation of the serum. The strains selected varied in their ability to produce lysis of blood suspensions and clearing of heated blood.

The strains tested (other than those which were antigenically similar to *V. choleræ*) were serologically individualistic. No antigenic similarity could be demonstrated among vibrios which corresponded as regards their action on blood-agar and heated blood-agar. Strains which differed in these respects were also antigenically individualistic.

Thus, vibrios isolated from cases of clinical cholera, from cholera-carriers

* I am indebted to Dr. W. W. Taylor, of the Physiology Department of Edinburgh University, for carrying out the spectroscopic examination.

and from water may be divided into four distinct groups by their lytic effect on blood suspensions and their action on blood-agar and heated blood-agar, viz :

	Hæmolysis in blood suspensions.	Clearing of heated blood.	Clearing of blood-agar.
I	−	−	−
II	+	+	+
III	+	−	+
IV	−	+	+

Note.—The results of clearing of blood-agar are due either to true hæmolysis, or to the effect indicated in the second column.

This method of classifying vibrios cannot be correlated with their serological characteristics. Strains which show strong serological relationships to the classical cholera strains fall into different groups in the above classification. Other strains belonging to each group are distinct antigenically from members of other groups and from other strains within the same group.

DISCUSSION.

As has long been recognized, *V. choleræ* (Koch) can be differentiated from the El Tor and paracholera vibrios by its failure to lyse blood when grown on blood-agar or when cultures are added to blood suspensions.

It has been demonstrated that the red cells of different animal species vary to some degree in their resistance to vibrio hæmolysins, and it appears likely that some of the apparent discrepancies in the literature referable to hæmolysin production by vibrios can be traced to the use of resistant red cells.

It is important to emphasize that in this work the terms "hæmolytic" and "non-hæmolytic" vibrios are used to designate strains which do or do not produce hæmolysis when fluid culture is added to a blood suspension.

The investigation of the production of clear areas around their growths by some strains of vibrios when grown on heated blood-media has led to interesting results. It has been shown that vibrios which produce clearing of heated blood-media also produce clear areas when grown on ordinary blood-agar, but may or may not produce lysis when cultures are added to blood suspensions. Similarly, fully grown cultures of El Tor and paracholera vibrios produce lysis of blood suspension and lysis also occurs in blood-agar cultures, but these organisms exert no clearing effect when grown on heated blood-medium. It would appear, therefore, that clearing of heated blood and true hæmolysis are due to two separate factors, although both properties may pertain to certain strains. Some vibrios, *e. g. V. choleræ* (Koch), exert no visible action on blood, either true hæmolysis or the "clearing" effect referred to.

An attempt was made to ascertain the chemical changes involved in the clearing of blood-agar. According to Van Loghem (1913), oxy-hæmoglobin can be demonstrated in the lysed zone produced by the El Tor vibrios, and he suggested that these organisms act on the envelopes of the red cells. Kovács (1926) claimed that those strains of *V. choleræ* which produce clearing of heated blood act on the hæmoglobin of the corpuscles. In this inquiry these observations were confirmed, and it was shown that the formation of alkali hæmatin was associated with the clearing of heated blood. The areas around colonies of El Tor and paracholera vibrios (Group III) grown on blood-agar presented a colour similar to lysed blood, whereas vibrios which cleared heated blood (Groups II and IV) when grown on blood-agar produced clear areas of a definite brown tint. The clear area surrounding colonies of El Tor and paracholera vibrios almost invariably extended further into the medium than similar areas formed by strains belonging to Groups II and IV.

The two factors concerned in the clearing of blood appear to be (1) a filterable hæmolysin as in the case of the El Tor and paracholera vibrios, and (2) a chemically active agent possibly of ferment nature, capable of splitting hæmoglobin into alkali hæmatin and other products. Certain vibrios apparently produce both filterable hæmolysin and this chemically active principle. In other strains the two properties are dissociated. The question has been considered whether the chemical alteration of hæmoglobin into various products, including alkali hæmatin, might result from alkali formation in the medium. Experiments were carried out to test the comparative alkali production by representative strains of the four vibrio groups which have been distinguished, but no appreciable differences in this respect were noted in plain peptone-water media. In view of the possible ferment nature of the agent concerned in the splitting of hæmoglobin, attempts are being made to obtain further information on this effect.

Table VII shows the sources of strains belonging to the various groups described above. Of 21 strains stated to have been isolated from "clinical cholera" in India, all types except the El Tor variety are represented. Three vibrios isolated from "carriers" did not produce hæmolysis or clearing of heated blood and were not agglutinated by an anticholera serum. Among the series of water vibrios examined, 5 types are represented. Of 97 strains of vibrios isolated in India, none were found to correspond to the classical El Tor strains.

In addition to the vibrios obtained from known sources, 51 strains collected from different laboratories in India were examined. It has been impossible to ascertain the original source of these strains.

Altogether 106 strains of vibrios were examined. Their classification among the four groups described is shown in the table.

The geographical relationships of the various groups is of some interest. The absence of the El Tor type among strains of Indian origin is noteworthy. Of 32 strains obtained from Madras, none corresponded to the paracholera vibrios of Mackie.

No relationship has been found between serological reactions and the differential characters elicited by tests for hæmolysis and clearing of heated blood-agar.

SUMMARY AND CONCLUSIONS.

1. The method of growing vibrios on blood-agar is unsuitable for determining their hæmolytic properties. For this purpose a fluid culture should be added to a suspension of ox or sheep blood.

2. Certain vibrios produce a clearing effect when growing on medium containing heated blood. These strains also clear blood-agar. They may not, however, prove hæmolytic when cultures are added to a blood suspension.

3. Vibrios which clear heated blood may or may not agglutinate with an anticholera serum.

4. The clearing of heated blood by vibrios is probably due to breaking down of hæmoglobin into several products, one of which appears to be alkali hæmatin.

5. The classical cholera vibrio, the El Tor strains and the paracholera vibrios do not clear heated blood.

6. Vibrios may be classified into 8 groups by the clearing of heated blood, lysis of blood suspension and agglutination with an anticholera serum. Strains isolated from cases of clinical cholera in India fall into 7 of these groups. The El Tor vibrios constitute the eighth group. Among 97 unselected strains of vibrios isolated in India, no strains of this group have been identified.

7. With the exception of strains which agglutinated with an anticholera serum, no serological relationship can be demonstrated among vibrios which reacted similarly when grown on heated blood or when cultures were added to blood suspensions.

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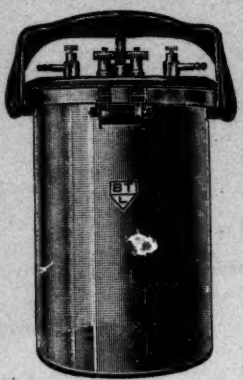

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